Pseudorabies virus-specific antibodies mask infected monocytes from immune recognition, induce a quiescent infection, and allow virus transmission to endothelial cells

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De waerheyt borrelt uyt
gelijck een sonneschijn;
De waerheyt, hoe het gaat,
wil niet begraven sijn.

Jacob Cats
(Nederlands dichter, 1577-1660)

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List of abbreviations

AACGL	antistof-afhankelijke complement-gemedieerde cellyse
ADCC	antibody-dependent cell-mediated cytotoxicity
ADCML	antibody-dependent complement-mediated cell lysis
AV	Aujeszky virus
Be	Becker
CNS	central nervous system
CSB	cytoskeleton-stabilizing buffer
C-SN	complement facilitated serum neutralization test
CTL	cytotoxic T-lymphocyte
DiI-Ac-LDL	1,1'-dioctadecyl-1-3,3,3',3'-tetramethylindo carbo-cyanine-perchlorate-acetylated low density lipoprotein
EBV	Epstein-Barr virus
EMA	ethidium mono-azide bromide
FCS	fetal calf serum
gB, gC,…	glycoprotein B, glycoprotein C,…
HCMV	human cytomegalovirus
HHV-6	human herpesvirus 6
HIV	human immunodeficiency virus
HSV	herpes simplex virus
IE	immediate early
IgG, IgM,…	immunoglobulin G, immunoglobulin M,…
IPMA	immunoperoxidase monolayer assay
Ka	Kaplan
LAT	latency-associated transcript
LFA-1	leukocyte function associated antigen-1
Mac-1	membrane associated component-1
MACS	magnetic antibodies cell separation
MMP	matrix metalloproteinases
m.o.i.	multiplicity of infection
NK	natural killer
p.Ab.a	post antibody addition

pAbs	polyclonal antibodies
PBS	phosphate buffered saline
PI	propidium iodide
p.i.	post inoculation
PRV	pseudorabies virus
RCA	regulators of complement activation
SK	swine kidney
SN	serum neutralization
TAP	transporter associated with antigen presentation
$TCID_{50}$	tissue culture infectious dose with a 50% endpoint
VLA-4	very late antigen-4
WB	washing buffer

1.

INTRODUCTION

1.1 Pseudorabies virus

1.1.1 Introduction

Pseudorabies virus (PRV) is a member of the *Alphaherpesvirinae* and is the causative agent of Aujeszky's disease in its natural host, the pig. The disease was first described in the beginning of the 19th century in cattle and was called "mad itch" because the symptoms resembled rabies very well. The causative agent, however, was only discovered in the early 20th century by the Hungarian physician Aujeszky (Aujeszky, 1902) and some years later, the disease was proven by filtration experiments to be caused by a virus (Schmitthofer, 1910). Most mammals, except higher primates, are susceptible for PRV. Of the susceptible animals, only pigs are known to survive an infection and therefore represent the natural reservoir of PRV (Mettenleiter, 1996). Whereas until the 1970s disease outbreaks occurred rather sporadically in pigs, an increase in case numbers and severity was reported from that time point on, probably correlated with an intensified pig husbandry (Gustafson, 1986). PRV causes a large spectrum of symptoms in its natural host with the age of the affected pig and the virulence of the infecting strain determining the onset and severity of the clinical signs. The symptoms consist of fever, lethargy, respiratory and neurological disorders, abortion and infertility (Wittmann & Rziha, 1989).

1.1.2 The virus

PRV, also designated suid herpesvirus type 1 (SHV-1) or Aujeszky's disease virus, belongs to the *Herpesviridae*, a family containing over 100 members, which are found both in warm- and cold-blooded vertebrates and invertebrates (Roizman, 1982). The herpesvirus family is divided into three subfamilies *Alpha (α)–, Beta (β)– and Gamma (γ)–herpesvirinae* (reviewed by Roizman, 1996). *Alpha-herpesvirinae* (including PRV) frequently have a broad host-range, a short replication cycle and establish latency in neuronal tissues. The *β-herpesvirinae* are characterized by a narrow host-range, a slow replication cycle and the frequent enlargement of infected cells (cytomegaly). These viruses can maintain a latent state in lymphoreticular cells. The host range of *γ-herpesvirinae* is almost exclusively restricted to one species and they replicate almost exclusively in either B- or T-lymphocytes. Latency is established in lymphoid tissues. Table 1 gives an overview of the three subfamilies of

the herpesvirus family with their genera and some of the best known members of each genus.

Table 1. Subfamilies, genera and some species of *Herpesviridae*

Subfamily	Genus	Species
α-herpesvirinae	Simplexvirus	human herpesvirus 1&2 (herpes simplex virus-1 &-2)
		bovine herpesvirus 2
	Varicellovirus	human herpesvirus 3 (varicella zoster virus)
		equid herpesvirus 1&4
		suid herpesvirus 1 (pseudorabies virus)
		bovine herpesvirus 1
		felid herpesvirus 1
		canid herpesvirus 1
	Marek's disease-like viruses	gallid herpesvirus 2
	Infectious laryngo-tracheitis-like viruses	gallid herpesvirus 1
β-herpesvirinae	Cytomegalovirus	human herpesvirus 5 (human cytomegalovirus)
	Muromegalovirus	murid herpesvirus 1
	Roseolovirus	human herpesvirus 6
	Lymphocryptovirus	human herpesvirus 4 (Epstein-Barr virus)
γ-herpesvirinae	Rhadinovirus	saimiriine herpesvirus 2
		bovine herpesvirus 4
		murid herpesvirus 4
		Ateline herpesvirus 2
		equid herpesvirus 2&5&7
	Ictalurid herpes-like viruses	Ictalurid herpesvirus 1

PRV, like most herpesviruses, is a complex virus of 150-180 nm in diameter and consists of four morphologically distinguishable structures (a schematic drawing of PRV is shown in Figure 1.) (reviewed by Mettenleiter, 1994). The interior of PRV consists of the *nucleoprotein* core, containing the linear double-stranded DNA of approximately 142 kbp and bound proteins. This nucleoprotein is enclosed in an icosahedral *capsid*, consisting of 162 capsomers (12 pentons, 150 hexons). The nucleoprotein and capsid form the nucleocapsid which is surrounded by the mostly amorphous *tegument* which is surrounded by the *envelope*, an irregularly shaped bilayer of phospholipids, in which different glycoproteins are embedded. For PRV, 11 glycoproteins have been characterized and are named according to the unified nomenclature based on their homology to herpes simplex virus 1 (HSV-1). All glycoproteins are constituents of the virion, except gG, which is secreted into the medium by infected cells. An overview of the different PRV glycoproteins with their most important functions can be found in Table 2.

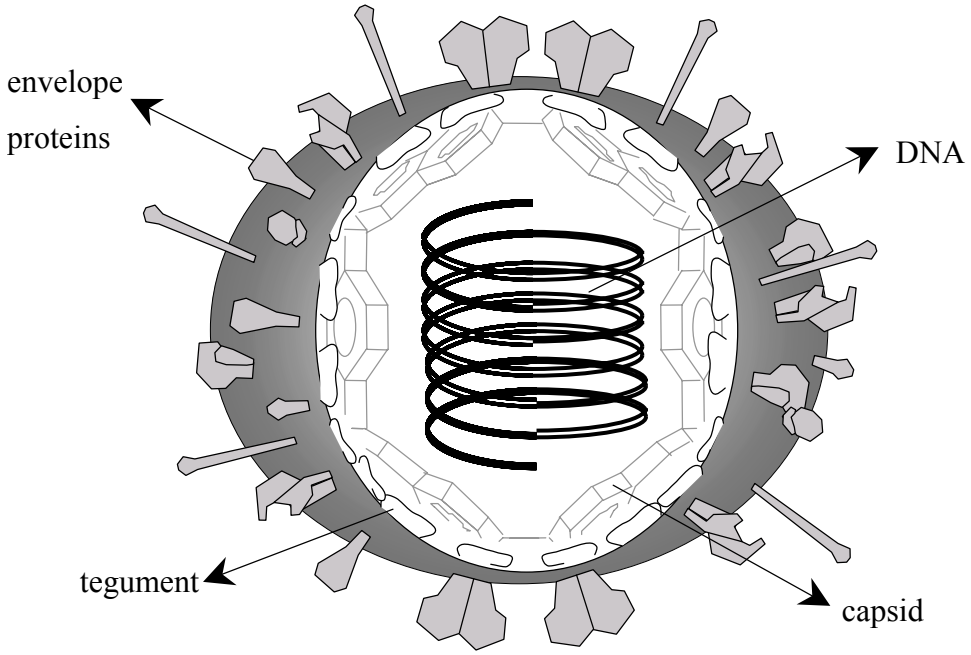

Figure 1. Schematic representation of PRV

Table 2. Overview of the PRV glycoproteins and their most important functions

glycoprotein	function	references
gB	Involved in attachment Essential for virus penetration, direct cell-to-cell spread and transneuronal spread Essential for efficient antibody-induced internalization of viral cell surface proteins	Sawitzky *et al.*, 1990 Rauh & Mettenleiter, 1991 Babic *et al.*, 1993 Favoreel *et al.*, 1999b
gC	Involved in initial non-essential binding Affects thermostability of the virus and virus release Binds with C3 of the complement cascade Major target protein for CTLs	Mettenleiter *et al.*, 1990 Schreurs *et al.*, 1988 Huemer *et al.*, 1992 Zuckermann *et al.*, 1990
gD	Essential for stable binding of virus to the host cell and subsequent fusion Essential for efficient antibody-induced internalization of viral cell surface proteins	Rauh & Mettenleiter, 1991 Peeters *et al.*, 1992a Favoreel *et al.*, 1999b
gE	Involved in cell-to-cell spread and virus egress Very important in neuronal spread of the virus (anterograde transport) gE displays low affinity Fc receptor activity	Mettenleiter *et al.*, 1987 Zsák *et al.*, 1989 Card *et al.*, 1992 Kritas *et al.*, 1995 Favoreel *et al.*, 1997
gG	Its function remains unknown, may have a role in cell-to-cell spread	Demmin *et al.*, 2001
gH	Essential for fusion of virion envelope with host's plasma membrane Essential for cell-to-cell and transneuronal spread	Peeters *et al.*, 1992b Babic *et al.*, 1996
gI	Forms with gE a functional entity gE-gI displays high affinity Fc receptor activity	Zuckermann *et al.*, 1988 Favoreel *et al.*, 1997
gK	Essential for virus egress Inhibits immediate re-infection of released virions	Klupp *et al.*, 1998
gL	Forms with gH a functional entity Essential for virus penetration, cell-to-cell spread and transneuronal spread	Klupp *et al.*, 1994 Klupp *et al.*, 1997 Flamand *et al.*, 2001
gM	Inhibitory effect on viral fusion	Klupp *et al.*, 2000
gN	Forms with gM a functional entity Involved in virus penetration	Jöns *et al.*, 1998

1.1.3 Interaction with the host cell

Infection of cells with PRV occurs either by free virions or by direct cell-to-cell spread from PRV-infected to adjacent non-infected cells. Although both processes are related, there are some differences e.g. gB and gH-gL are essential for both penetration of free virions and direct cell-to-cell spread, whereas gD is only essential for infection by free virions (Rauh & Mettenleiter, 1991; Peeters *et al.*, 1992a; Peeters *et al.*, 1992b; Heffner *et al.*, 1993). A schematic representation of the replication cycle of PRV in its host cell can be found in Figure 2.

Attachment: glycoprotein C is the major glycoprotein involved in primary attachment by binding to heparan sulfate-carrying proteoglycans on the cell membrane (Mettenleiter *et al.*, 1990). This heparin-sensitive, labile attachment is followed by a heparin-resistant, stable secondary interaction, mediated by gD which binds to different isoforms of nectin-1 and nectin-2, also designated 'Herpesvirus entry mediators' (Hve) (Karger & Mettenleiter, 1993; Warner *et al.*, 1998; Haarr *et al.*, 2001).

Penetration: after a pH independent fusion of the viral envelope with the plasma membrane, capsids are released in the cytoplasm of the host cell (Spear, 1993). Glycoproteins gB, gD, gH and gL are essential for this process (Rauh & Mettenleiter, 1991; Peeters *et al.*, 1992a; Peeters *et al.*, 1992b; Klupp *et al.*, 1997), whereas gM and gN are merely modulatory for virus penetration (Dijkstra *et al.*, 1996; Jöns *et al.*, 1998).

Transport into the nucleus: the mechanism of transport to the nucleus and subsequent release of the genomic material in the nucleus is still poorly understood. The transport of the capsids to the nuclear membrane is assumed to occur along microtubules and it is suggested that DNA is extruded from the capsids and enters the nuclear pore via the vertex region (Granzow *et al.*, 1997). More recently, UL25, a minor capsid protein, has been identified as being important during this transport to the nucleus (Kaelin *et al.*, 2000).

Intranuclear events: upon entry of the viral genome in the nucleus, the linear DNA circularises, and transcription is initiated. This transcription is regulated in a cascade-

like fashion (reviewed by Mettenleiter, 1994). “Immediate-early genes” are expressed first and encode transcription factors, necessary for efficient transcription of early genes. For PRV, there is only one gene with immediate-early kinetics and it encodes the protein IE180 (Cheung *et al.*, 1990). IE180 is a potent activator of “early genes”, which are expressed before DNA replication and encode proteins like thymidine kinase (McGregor *et al.*, 1985) and ribonucleotide reductase (de Wind *et al.*, 1993), important for mediating DNA replication. Transcription of “early-late genes” starts before DNA replication but reaches its maximum level after DNA replication has been initiated. This DNA replication proceeds mainly via a rolling circle mechanism leading to long head-to-tail concatameric DNA molecules which need to be cleaved into unique length genomes before or during packaging into capsids (Ben-Porat & Kaplan, 1985). Transcription of the “late genes” starts after DNA replication and these late genes encode several structural capsid and envelope (glyco) proteins. It has to be pointed out, however, that not all viral genes can be easily classified as ‘immediate early’, ‘early’ or ‘late’ genes.

Viral egress: capsids, containing the viral genome, leave the nucleus by budding through the inner nuclear membrane and this first viral envelope appears to contain no (mature) glycoproteins (Granszow *et al.*, 1997). This primary envelope is then lost by fusion with the outer nuclear membrane, resulting in the release of naked capsids into the cytoplasm. A secondary envelopment process takes place in the trans-Golgi area where tegument is included and viral glycoproteins are clearly present in this secondary envelope (Granszow *et al.*, 1997). The net result of this budding event is the presence of complete virus particles within exocytic vesicles which then fuse with the plasma membrane, thereby releasing the virus particles into the extracellular space. The glycoproteins gC and gE-gI seem to be required for efficient virus release (Mettenleiter *et al.*, 1987; Zsak *et al.*, 1989) and gK may prevent immediate re-infection of the released virions (Klupp *et al.*, 1998).

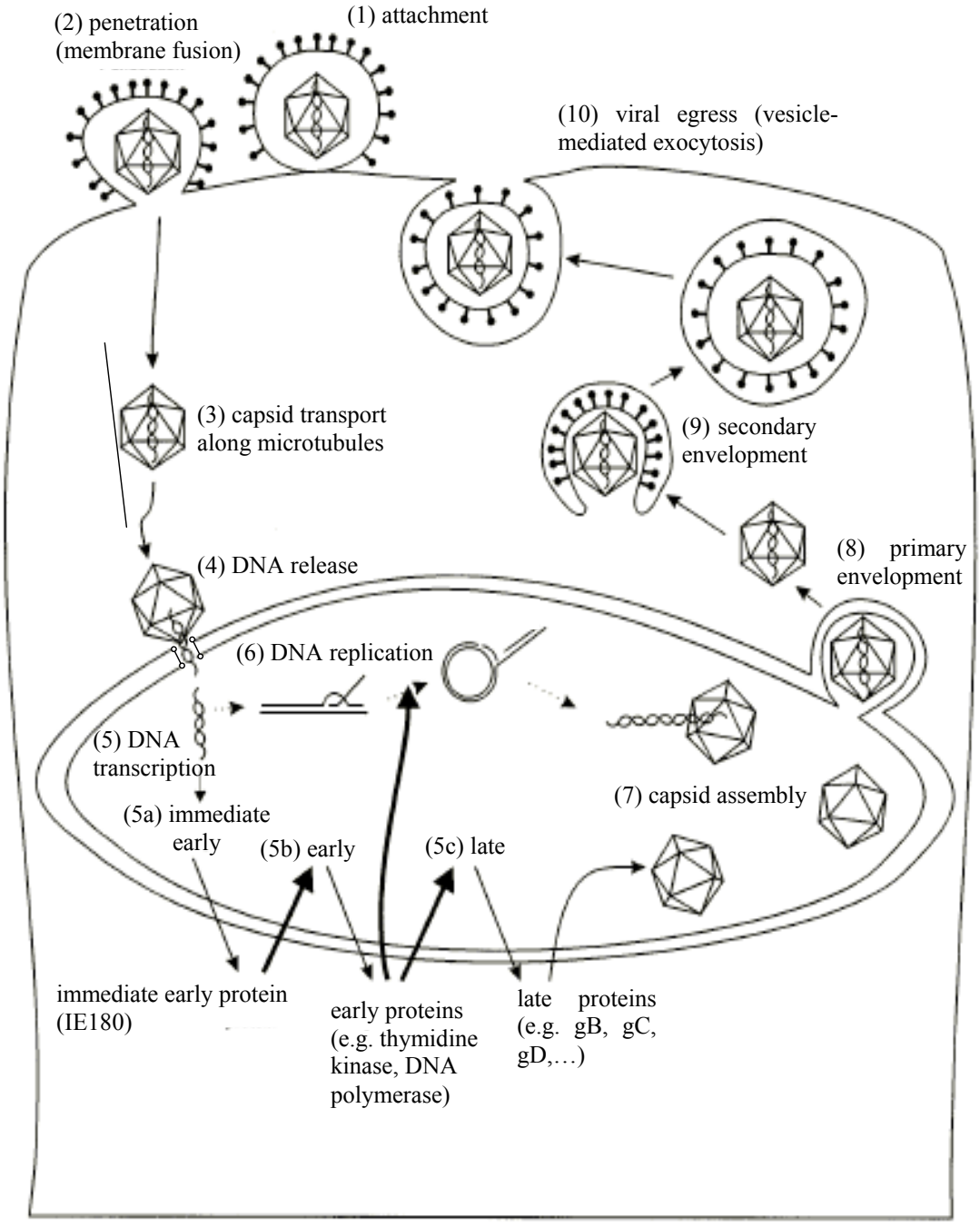

Figure 2. Schematic representation of the PRV replication cycle in the host cell (adapted from Mettenleiter, 2000).

1.2 Pathogenesis of pseudorabies virus infections

1.2.1 Introduction

Pigs are the natural host in which the virus circulates, causing respiratory problems, central nervous disorders and reproductive failures. Other animal species, except higher primates, may become infected when they are exposed to high quantities of PRV excreted by PRV-infected pigs or present in organic material coming from PRV-infected pigs. Infection of these animals is lethal with central nervous disorders and death within 2 days. Since infected non-porcine animals usually die without having shed PRV, they are of minor importance for virus transmission to contact animals.

This sub-chapter will highlight the most important data on the pathogenesis of PRV infection, both in naive and immune pigs, since this information (especially the pathogenesis in immune animals) is important for the aims of the research performed in these studies.

1.2.2 PRV infection in naive pigs

Under natural conditions, the primary site of replication is situated in the respiratory tract, including nasal cavity, tonsils, pharynx and lungs (Sabo *et al.*, 1969; Baskerville, 1973; Miry & Pensaert, 1989a). Due to the broad range of susceptible host cells, the short replication cycle and the direct cell-to-cell spread, PRV is able to penetrate very quickly through the epithelium and underlying connective tissue. At 24h post inoculation, PRV is already found in epithelial cells, fibrocytes and nerve cells of the nasal cavity; at 48h post inoculation, viral antigens are detected in large plaques in the epithelium and connective tissues, including nerves and endothelial cells of blood vessels (Wittmann *et al.*, 1980). From these primary sites, PRV spreads to distant secondary replication sites via lymph, blood and nerves. Viraemia occurs both under a cell-free and cell-associated form (with monocytes as most susceptible for PRV infection) (Nauwynck & Pensaert, 1995). Virus replication at these secondary sites is detected starting from 48h post inoculation in draining lymph nodes, olfactory bulb, medulla, spleen, kidneys, ovaries and uterus (Sabo *et al.*, 1969; Wittmann *et al.*, 1980). This replication, however, is less extensive than in the primary sites and is clearly reduced with the onset of the immune response.

The central nervous system (CNS) is an important secondary site of PRV replication, causing nervous disorders in neonatal pigs. The CNS is reached by PRV via some of the cranial nerves such as the olfactory and trigeminal nerves (McFerran & Dow, 1965; Sabo *et al.*, 1969, Wittmann *et al.*, 1980). More detailed studies revealed that after intranasal inoculation of pigs, PRV travels from the nasal mucosa via the nerves of both the olfactory and trigeminal nervous pathways towards the ganglia in a non-infectious form, then replicates in the neuronal cell bodies after which infectious virus is transported towards tissues of the CNS or back towards the nasal mucosa. This transport (away from neuronal cell bodies) is also called anterograde transport, with gE and gI as important viral proteins during this process (Kritas *et al.*, 1994a; Kritas *et al.*, 1994b; Kritas *et al.*, 1995).

The genital organs also are important secondary sites of PRV infection. It is assumed that these organs are reached via blood or nerves (McFerran & Dow, 1965) (and to a much lesser extent via PRV-contaminated sperm, Bolin *et al.*, 1985). Replication of PRV has been described in the mesorchium of boars, leading to a severe periorchitis with a temporal or permanent infertility as a result (Miry & Pensaert, 1989b). Replication has also been described in the ovaria and the uterus of sows (Hsu *et al.*, 1980). When a pregant sow becomes infected with PRV in the first trimester of gestation, fetuses are resorbed and the sow returns to estrus. A transplacental PRV infection in the second or third trimester results in abortion or stillborn pigs (Kluge *et al.*, 1999). Reproductive failure can occur in 20% of the pregant sows (Kluge & Maré, 1974).

1.2.3 PRV infection in immune pigs

The immune response against PRV starts at 7 days post inoculation and consists of the action of multiple defence mechanisms leading to elimination of the virus. Immunity can be established after a natural infection or after vaccination. The immune mechanisms are roughly subdivided in an early aspecific defence and a late specific defence. The *early aspecific defence* consists of activities, which are not specifically directed against PRV. Important reactions are the production of interferon (Wittmann *et al.*, 1980; Pol, 1990; Nauwynck & Pensaert, 1995), lysis of PRV-infected cells via activation of the alternative/lectin-binding complement pathway (in the absence of antibodies) (Kimman *et al.*, 1992a) and the spontaneous cell-mediated cytotoxicity (predominantly mediated by macrophages, neutrophils and natural killer

cells) (Martin & Wardley, 1984; Wittmann *et al.*, 1985). The *late specific defence* consists of PRV-specific actions including PRV-specific antibodies and PRV-specific cytotoxic T-lymphocytes (CTLs). PRV-specific IgM and IgA are found in all fluids, whereas IgG1 and IgG2 antibodies are only present in serum (Rodak *et al.*, 1987; Kimman *et al.*, 1992a). These antibodies will bind envelope glycoproteins, expressed on virions as well as on the plasma membrane of PRV-infected cells, thereby rendering these virions and/or infected cells recognizable for activation of the classical pathway of complement and antibody-dependent cell-mediated cytotoxicity (mediated by macrophages, neutrophils and natural killer cells) (Ashworth *et al.*, 1979; van Oirschot *et al.*, 1984; Wittmann *et al.*, 1985; Kimman *et al.*, 1992a). PRV-specific CTLs, also designated $CD8^+$ T cells, are found in the blood and lymphoid organs of PRV-infected pigs, together with PRV-specific T-helper lymphocytes or $CD4^+$ T cells (Kimman *et al.*, 1992b; Kimman, 1994). The viral glycoproteins gB and gC are major target proteins for the PRV-specific CTLs (Zuckermann *et al.*, 1990; Kimman, 1994).

Immunity after a PRV-infection induces a short virological but a long clinical protection. Following a reinfection at one month after the primary infection, a restricted replication of PRV can be observed in tonsils and lungs (alveoli and alveolar macrophages) but not in the nasopharynx. A time interval of 3 months results in virus replication of both the upper and lower respiratory airways (Sabo & Blaskovic, 1970; Miry & Pensaert, 1989a).

Protection against PRV after vaccination is less efficient compared to the protection induced by a natural infection. Reinfection at one month after vaccination (with an inactivated vaccine parenterally or an attenuated vaccine intranasally) results not only in PRV replication in the lungs, but also in the pharynx, tonsils and nasal cavity (Wittmann *et al.*, 1980; Miry & Pensaert, 1989a). Moreover, despite the presence of a vaccination-induced immunity (using an inactivated vaccine), a cell-associated viraemia can still occur which allows PRV to reach the inner organs (Wittmann *et al.*, 1980). Such a viraemia generally does not cause problems. However, abortion has been reported on well-vaccinated farms (Dieuzy *et al.*, 1987; de Mûelenaere & Pensaert, 1989) and occurs as the result of cell-associated transplacental spread and intrafetal replication (Nauwynck & Pensaert, 1992). Porcine blood monocytes have been shown to be essential to transport the virus via the blood

to the pregnant uterus in vaccination-immune pigs (Nauwynck & Pensaert, 1992; Nauwynck & Pensaert, 1995). Exactly how these infected monocytes survive in the blood in the presence of an immunity is still poorly understood, although recently, a process of antibody-induced clearance of viral cell surface proteins has been demonstrated which may lower the susceptibility of PRV-infected cells towards antibody-dependent cell lysis (Favoreel *et al.*, 1999*a*).

1.3 Immune evasion strategies of pseudorabies virus

1.3.1 Introduction

Herpesviruses are masters in avoiding destruction by the immune system. All members of this family are capable of replicating in immune animals and establishing long-term persistence. Many properties of these viruses minimize the chance of elimination by complement, phagocytes, cytotoxic T-lymphocytes and/or natural killer cells. This sub-chapter will give a brief overview on the recent knowledge of the specific immune evasion strategies used by PRV.

1.3.2 Evasion of complement-mediated lysis

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Complement consists of an interacting set of enzymes which, upon activation, give rise to a cascade of reactions, finally resulting in destruction of invading microorganisms and infected cells. Evasion of this complement activation system is of particular importance for viruses since it makes part of both the innate immunity (alternative, antibody-independent complement activation and mannan-binding lectin pathway) and the adaptive immunity (classical, antibody-dependent complement activation).

gC complement binding

PRV, as well as the other members of the *α-herpesvirinae*, encodes the conserved viral glycoprotein gC, a non-essential glycoprotein known to play a role in virus attachment, virus release and virulence (Schreurs *et al.*, 1988; Mettenleiter *et al.*, 1990; Herold *et al.*, 1991). Besides that, gC has also been reported to bind C3, the pivotal component of the alternative complement cascade, with highest affinity to C3 of the natural host (Friedman *et al.*, 1984; Huemer *et al.*, 1993). This alternative pathway of the complement cascade can become activated in the absence of antibodies and is therefore very important during early infection when humoral immunity is not yet achieved (Tizard, 1992). Binding of gC on PRV virions with the complement component C3 presumably results in inhibition of further downstream

events of this alternative activation of complement (Huemer *et al.*, 1992; Huemer *et al.*, 1993), thereby protecting PRV from complement-mediated lysis (Maeda *et al.*, 2002).

Virion incorporation of regulatory control proteins of complement activation (RCAs)

Complement activation is a potentially dangerous system and must therefore be very carefully regulated. Normal mammalian cells are protected from complement-mediated destruction by the activities of "regulatory control proteins of complement activation" (RCAs), which are restricted predominantly to complement of the same species (Tizard *et al.*, 1992). In a recent study, it was demonstrated that PRV grown in a porcine cell line, was protected against the actions of porcine complement, whereas the same PRV strain grown in a rabbit cell line, was extremely sensitive to lysis by porcine complement (Maeda *et al.*, 2002). This resistance of the porcine cell line-derived PRV strain was thought to occur via incorporation of RCAs into the viral envelope during egress from the host cell. However, the RCAs responsible for this protection have not been identified to date.

1.3.3 Evasion of antibody-dependent cell lysis

PRV-infected blood monocytes express viral envelope proteins on their plasma membrane (Favoreel *et al.*, 1999b). PRV-specific antibodies will bind to these viral glycoproteins, which should induce antibody-dependent lysis of the infected cells (Sissons & Oldstone, 1980). This, however, seems to occur inefficiently during a PRV infection, since PRV-infected monocytes are able to survive in the blood of vaccinated animals (Nauwynck & Pensaert, 1992). Several strategies have been described which may help to explain the inefficient antibody-dependent lysis of PRV-infected cells.

gE-gI Fc receptor activity

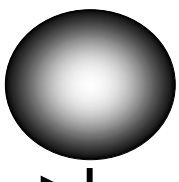
The expression of a viral Fc receptor, consisting of the viral gE-gI complex, has been very intensively studied for herpes simplex virus (HSV), the prototypical member of the *α-herpesvirinae* (Favoreel *et al.*, 2003). gE-gI of PRV has also been shown to display Fc receptor activity (Favoreel *et al.*, 1997). Expression of a viral Fc receptor may interfere with efficient antibody-dependent immune effectors via (i) binding of non-immune immunoglobulin G (IgG) to the Fc receptor, expressed on virus or virus-

infected cells, by which access of viral-specific immune IgG is sterically hindered (Dowler & Veltri, 1984) and (ii) "antibody bipolar bridging", consisting of simultaneous binding of the Fab sides of an antibody to viral proteins in the viral envelope or on the surface of infected cells, and of the Fc side of the same antibody to viral Fc receptors (Frank & Friedman, 1989), resulting in inefficient complement-mediated neutralization and antibody-dependent cellular cytotoxicity.

Although not yet examined, PRV gE-gI Fc receptor activity may lower the susceptibility towards antibody-dependent cell lysis.

Antibody-induced clearance of viral proteins in PRV-infected cells

A schematic representation of antibody-induced clearance of viral cell surface proteins can be found in Figure 3. This process was first described for PRV-infected swine kidney (SK)-cells and consisted of antibody-induced aggregation (patching), capping and shedding of the antigen-antibody complexes from the cell surface (Favoreel *et al.*, 1997). More detailed studies revealed that microtubules, a component of the cellular cytoskeleton, are of critical importance during the initial polarization of the patches and the formation of an early cap; the development of this early cap into a more pronounced cap was shown to be actin-dependent (Favoreel *et al.*, 1997). Viral glycoprotein gE was found to have a dual role during this process. First, the gE-mediated Fc receptor function was found to be necessary for efficient patching of antigen-antibody complexes (Favoreel *et al.*, 1997). Second, two tyrosine amino acid residues in the cytoplasmic tail of gE were found to be crucial for efficient polarization of the patched antigen-antibody complexes, possibly by mediating a signal transduction event (Favoreel *et al.*, 1999*b*). Clearance of antigen-antibody complexes from the cell surface could also be demonstrated in PRV-infected monocytes (Favoreel *et al.*, 1999*a*), the natural carrier cell of PRV in the blood of vaccinated animals (Nauwynck & Pensaert, 1992). Here, clearance consisted of clustering and subsequent internalization of the antigen-antibody complexes. Using PRV mutants, viral glycoproteins gB and gD were shown to be indispensable for efficient internalization (Favoreel *et al.*, 1999*a*). Recently, it was demonstrated that a single tyrosine residue in the cytoplasmic tail of gB is crucial for efficient internalization, possibly by linking gB to endocytosis adaptor protein complexes (AP-



Monocyte or SK cell

Figure 3. Schematic representation of antibody-induced clearance of viral cell surface proteins in pseudorabies virus-infected cells (adapted from Favoreel *et al.*, 2000)

2) as a first step in the formation of clathrin-coated endocytosis vesicles (Favoreel *et al.*, 2002). Although the mechanism of clearance depends on the cell type infected by PRV, both clearance mechanisms finally result in PRV-infected cells without visually detectable levels of antigen-antibody complexes on the plasma membrane. Although not yet examined, this mechanism may lower the susceptibility of PRV-infected cells towards antibody-dependent cell lysis.

1.3.4 Evasion of major histocompatibility complex (MHC) class I-dependent cell lysis

MHC class I glycoproteins are expressed on the plasma membrane of almost all nucleated cells and strictly control the activation of cytotoxic T-lymphocytes (CTL) and natural killer (NK) cells. These MHC class I molecules are produced in the endoplasmatic reticulum, become loaded with either a cellular or a viral peptide (after degradation in the cytoplasm by proteasome) and are finally transported to the plasma membrane. If the peptide originates from a cellular protein, CTLs will ignore the cell, if the peptide is derived from a foreign (e.g. viral) protein, specific CTLs will recognize this MHC class-I complex, triggering the CTLs to lyse or cure the infected cell (Harper, 1994; Pamer & Cresswell, 1998, Guidotti & Chisari, 2000).

PRV has been reported to down-regulate the porcine class I molecules on PRV-infected cells (Mellencamp *et al.*, 1991). One ore more early proteins of PRV appear to be responsible for this down-regulation by interfering with the peptide transport activity of 'the Transporter Associated with Antigen Presentation' (TAP) (Sparks-Thissen & Enquist, 1999; Ambagala *et al.*, 2000). Whether this downregulation of MHC class-I lowers the susceptibility of infected cells towards CTL's has not yet been examined. Besides this process, Favoreel *et al.* (1999b) demonstrated that during the process of antibody-induced internalization of viral cell surface proteins in PRV-infected monocytes (as described above), several cellular plasma membrane proteins, including MHC class-I molecules, are passively co-internalized. Moreover, it was shown that this co-internalization of MHC class-I complexes reduced the susceptibility of PRV-infected monocytes to lysis by cytotoxic T-lymphocytes (Favoreel, 1999).

1.3.5 Evasion of apoptosis

Apoptosis, also designated host cell suicide or programmed cell death, of an infected cell can be stimulated directly by replication of the virus in the cell or indirectly by CTLs and NK cells, which secrete cytotoxic cytokines such as tumor necrosis factors, perforine and granzyme proteins, or the Fas ligand. Such a premature cell death can limit the time available for the production of new virions and interrupt cycles of latency and reactivation used by persistent viruses (Dockrell, 2001). It is known that many viruses have acquired anti-apoptotic genes, which have been reported to render infected cells resistant to apoptosis (Thomson, 2001), but the PRV genome has to date not been reported to encode proteins with anti-apoptotic functions, although it has to be mentioned that some PRV proteins display amino acid sequence similarities to known HSV anti-apoptotic proteins like Us3, Us5 and LAT (Jerome *et al.*, 1999; Perng *et al.*, 2000; Nishiyama *et al.*, 2002). In a recent study by Aleman *et al.* (2001) it was shown that PRV is able to block apoptosis of the majority of infected trigeminal ganglionic neurons during an acute infection of swine. The surrounding inflammatory cells on the other hand, did undergo apoptosis implying a suppression of cell-mediated immunity following PRV infection. More research, however, is necessary to get a better understanding of the underlying mechanisms of these interesting observations.

1.3.6 Latency

A characteristic of herpesvirus infections is the ability of the virus to establish a lifelong persistence in their host after the acute phase of infection, designated as viral latency. The major site of PRV latency, like HSV latency, consists of the trigeminal ganglionic neurons and other sites, such as the olfactory bulb, tonsils, and to a lesser extent, cells of the haematopoietic system, have also been suggested to contain latent PRV (Wittmann *et al.*, 1983; Mettenleiter, 1994; Cheung, 1995; Balasch *et al.*, 1998). During latency, viral DNA persists, but infectious virus is not produced. In this state of virus infection, only a very specific subset of viral genes is transcribed, the so-called latency-associated transcripts (LATs), usually at a very low level, without detectable protein expression, making latently infected cells very hard, if not impossible, to be recognized by the immune system (reviewed by Jones, 1998). Upon certain stimuli, such as stress, the virus can reactivate from latency, which may result in virus production, excretion and spread. Recently, it has been shown that for PRV,

the LAT gene can also be transcriptionally active during a productive, lytic infection (Jin & Scherba, 1999).

1.4 Aims of the study

It has been reported earlier that in the presence of a vaccination-induced immunity, pseudorabies virus (PRV) may still replicate in the respiratory tract and draining lymph nodes, resulting in a restricted viraemia (Wittmann *et al.*, 1980). This limited replication in immune animals generally does not cause problems. However, abortion may occasionally occur as a result of cell-mediated transplacental spread and intrafetal replication (de Mûelenaere & Pensaert, 1989; Nauwynck & Pensaert, 1992). Porcine blood monocytes have been shown to be essential to transport the virus to the pregnant uterus in vaccination-immune pigs (Nauwynck & Pensaert, 1992; Nauwynck & Pensaert, 1995). These infected monocytes are able to spread in the blood of vaccinated animals, which implies that these cells can temporary or permanently avoid efficient lysis by the immune system. Recently, a process has been described how these PRV-infected monocytes *in vitro* may avoid efficient antibody-dependent lysis: addition of PRV-specific antibodies to PRV-infected monocytes results in aggregation of the majority of membrane-bound viral cell surface proteins, followed by internalization of these antigen-antibody complexes (Favoreel *et al.*, 1999b). Although not yet examined, this process may interfere with efficient antibody-dependent cell lysis. This potentially new immune evasion mechanism used by PRV is fast and efficient and is mediated by the viral proteins gB and gD (Favoreel *et al.*, 1999b; Favoreel *et al.*, 2002).

The aims of the work described in this thesis were to investigate in detail *in vitro* the underlying mechanism of this antibody-induced internalization process and the consequences of this process with regard to susceptibility of the PRV-infected cells towards antibody-dependent cell lysis, viability of and viral protein expression in the infected cells, and spread of the virus to internal organs.

First, it was evaluated whether the antibody-induced internalization process indeed can have an effect on the efficiency of antibody-dependent cell lysis. Therefore, it was investigated if this potential immune-evasion mechanism renders the PRV-infected monocytes less susceptible towards antibody-dependent complement-mediated cell lysis (Chapter 2).

Next, the underlying mechanism of this antibody-induced internalization process was studied in more detail. Viral proteins gB and gD are crucial to efficiently initiate the process, and the aim of this part of the thesis was to examine if the subsequent steps necessary to complete the process are also virus-mediated or, alternatively, mediated by the cellular components known to be important in physiological endocytosis processes. (Chapter 3).

The next aim of the study was to evaluate the long term fate of PRV-infected monocytes with internalized viral cell surface proteins in the continuous presence of PRV-specific antibodies with regard to cell viability and viral protein expression (Chapter 4).

The aim of the final part of the study was to evaluate how PRV-infected monocytes with internalized viral cell surface proteins may transmit virus to vascular endothelial cells in the presence of neutralizing antibodies (Chapter 5).

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ANTIBODY-INDUCED INTERNALIZATION OF VIRAL GLYCOPROTEINS AND GE-GI FC RECEPTOR ACTIVITY PROTECT PSEUDORABIES VIRUS-INFECTED MONOCYTES FROM EFFICIENT COMPLEMENT-MEDIATED LYSIS

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Summary

Pseudorabies virus (PRV)-infected blood monocytes are able to transport the virus throughout the body of vaccination-immune pigs. PRV-infected monocytes express viral proteins in their plasma membrane that can be recognized by virus-specific antibodies. Recently, it has been shown that addition of PRV-specific polyclonal immunoglobulins to PRV-infected monocytes at 37 °C induces internalization of the majority of plasma membrane-expressed viral proteins. This study investigated whether this process may interfere with efficient antibody-dependent complement-mediated lysis (ADCML) of PRV-infected monocytes *in vitro*. Therefore, an ADCML assay was set up, using PRV-infected porcine blood monocytes, porcine anti-PRV antibodies and porcine complement, to simulate the *in vivo* situation. It was shown that cells with internalized viral glycoproteins are significantly less susceptible toward ADCML than cells that did not undergo internalization ($p < 0.005$): 47% of the cells without internalization of viral cell surface proteins were lysed by ADCML, compared to only 12% of the cells that had undergone internalization of viral cell surface glycoproteins. Furthermore, it was shown (i) that the PRV gE-gI complex, which, like certain other alphaherpesvirus orthologues, possesses IgG-binding capacity, aids in avoiding efficient ADCML of PRV-infected monocytes and (ii) that the efficiency of PRV gE-gI-mediated evasion of ADCML can be decreased by the presence of gE-gI specific antibodies.

Introduction

Pseudorabies virus (PRV), a member of the subfamily *Alphaherpesvirinae*, causes Aujeszky's disease in its natural host, the pig. Clinical signs depend on the age of the pig and are characterised by nervous signs in newborn pigs, respiratory disorders in fattening pigs and reproductive failure in sows. Abortion may be an important consequence of PRV infection in pregnant sows (Pensaert & Kluge, 1989). In the presence of a vaccination-induced immunity, PRV may still replicate in the respiratory tract and draining lymph nodes, resulting in a restricted viraemia (Wittmann *et al.*, 1980). The limited replication in immune animals generally does not cause problems. However, abortion may occur as a result of cell-mediated transplacental spread and intrafetal replication. It has been shown that blood monocytes are essential to transport the virus via the blood of vaccination-immune pigs to different internal organs, including the pregnant uterus (Nauwynck & Pensaert, 1992; Nauwynck & Pensaert, 1995a), but little is known on exactly how these infected monocytes can survive in the presence of virus-neutralizing antibodies.

PRV-infected monocytes (and PRV-infected cells in general) express viral envelope proteins on their plasma membrane (Favoreel *et al.*, 1999). Antibodies bind to these viral cell surface proteins which should induce antibody-dependent lysis of the infected cells (Sissons & Oldstone, 1980). A major component of the antibody-dependent immune system is the complement cascade (reviewed by Sim & Dodds, 1997). Complement components bind to the Fc region of antibodies, which leads to a cascade of events and results finally in lysis of antibody-covered infected cells (antibody-dependent complement-mediated cell lysis = ADCML) (reviewed by Müller-Eberhard, 1984).

Recently, we described a process of how PRV-infected monocytes may avoid efficient lysis by ADCML. Addition of PRV-specific antibodies to PRV-infected monocytes *in vitro* results in aggregation of the majority of membrane-bound viral proteins, followed by internalization of these glycoprotein-antibody complexes (Favoreel *et al.*, 1999). The use of wild type PRV and isogenic deletion mutants showed that the viral glycoproteins gB and gD play a crucial role during this antibody-induced internalization process (Favoreel *et al.*, 1999; Van de Walle *et al.*, 2001). Recently, it was shown that a tyrosine-based motif in the cytoplasmic tail of

gB is crucial for the correct functioning of gB during the internalization process (Favoreel *et al.*, 2002). The internalization process is fast and efficient and leaves the infected monocyte with only few viral protein-antibody complexes on its surface. The purpose of the current study was to create an ADCML assay *in vitro* to investigate whether this antibody-induced clearance of the majority of the plasma membrane-anchored viral proteins results in inefficient ADCML of PRV-infected monocytes.

Furthermore, we also tested the importance of another potential strategy of PRV to resist ADCML: glycoprotein complex gE-gI Fc receptor activity. For herpes simplex virus (HSV), it has already been shown that this gE-gI Fc receptor binding is important for evasion of the antibody-dependent components of the immune system *in vitro* and *in vivo* (Johnson *et al.*, 1988; Frank & Friedman, 1989; Nagashunmugam *et al.*, 1998). PRV gE-gI has also been shown to display Fc receptor activity (toward porcine IgG) (Favoreel *et al.*, 1997), but its role in antibody-dependent immune evasion had not yet been studied. Therefore, besides studying the effect of antibody-induced internalization of viral cell surface glycoproteins on ADCML efficiency, we also investigated whether PRV gE-gI binding capacity interferes with efficient ADCML of PRV-infected monocytes.

Materials and Methods

Virus strains. PRV strains 89V87, NIA3, Becker (Be) and its gE-gI deletion mutant [$Be_{gE\text{-}gI\ null}$] and Kaplan (Ka) and its gE-gI deletion mutant [$Ka_{gE\text{-}gI\ null}$] were used. All strains have been described earlier (Kaplan & Vatter, 1959; McFerran & Dow, 1975; Mettenleiter et al., 1987; Nauwynck et al., 1992; Whealy et al., 1993; Mulder et al., 1994).

Antibodies. In most experiments, unlabeled or FITC-labeled protein A-purified IgG antibodies were used, derived from a PRV (89V87) inoculated pig, originating from a PRV-negative farm (Nauwynck *et al.*, 1995b). When mentioned, protein A-purified IgG antibodies were used derived from convalescent sera, which were obtained from pigs, originating from a PRV-negative farm, at 21 days after inoculation with 10^6 $TCID_{50}$ PRV (NIA3, Ka or $Ka_{gE\text{-}gI\ null}$). All purified antibodies, at a standardized concentration of 0.9 mg/ml IgG, had a titer between 24 and 64 with a complement facilitated serum neutralization test (C-SN) (Bitsch & Eskilden, 1982) and a titer between 160 and 640 with an immunoperoxidase monolayer assay (IPMA).

Porcine complement. Serum from a pig, originating from a PRV-negative farm (C-SN titer of < 2), was used as a source for porcine complement.

Isolation of porcine blood monocytes. PRV-negative pigs were used as blood donors. Blood was collected from the *vena jugularis* on heparin (15U/ml) (Leo, Zaventem, Belgium). Blood mononuclear cells were separated on Ficoll Paque® (Amersham Pharmacia Biotech AB, Uppsala, Sweden) following the manufacturer's instructions. Mononuclear cells were then resuspended in medium (A), based on RPMI-1640® (Gibco BRL, Life Technologies Inc., Paisley, Scotland) and supplemented with 10% fetal bovine serum (FBS), 0.3 mg/ml glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.1 mg/ml kanamycin, 1 mM sodium pyruvate, 1% non-essential amino acids 100x (Gibco BRL) and 10 U/ml heparin. Afterwards, cells were seeded on 4-well multidish (Nunc A/S, Roskilde, Denmark) at a concentration of $2.5x10^6$ cells/ml and cultivated at 37°C with 5% CO_2. At 24 h post-

seeding, non-adhering lymphocytes were removed by washing the 4-well multidish three times with RPMI-1640 as described before (Favoreel *et al.*, 1999). Purity of monocytes was always ≥70%, as assessed by flow cytometric analysis using the monocyte marker 74.22.15 (Pescovitz *et al.*, 1984).

Inoculation of blood monocytes. Monocytes were inoculated with different PRV strains at a multiplicity of infection (m.o.i.) of 10 in 0.5 ml medium (A) without heparin (Favoreel *et al.*, 1999). Cells were further incubated at 37°C with 5% CO_2. For all strains used and for all experiments, between 80 and 90% of the monocytes were infected and less than 5% of the non-monocytes were infected.

Antibody-dependent complement-mediated cell lysis (ADCML) assay. In general, at 13 h post inoculation (p.i.) with PRV, cells were centrifuged for 10 min at 500xg, washed, and resuspended in medium (A). Cells were incubated for 1 h at 37°C with IgG antibodies derived from porcine PRV-specific polyclonal hyperimmune serum (0.9 mg/ml, except where indicated otherwise). Cells were washed twice in RPMI-1640®, and incubated for 1h at 37°C with porcine complement (a 5% dilution in medium (A), except where indicated otherwise). Except where mentioned otherwise, 50 µg/ml of genistein (Sigma Chemical Company, St. Louis, Missouri, USA) was added 45 minutes before antibody addition and also during antibody and complement incubation. Genistein inhibits tyrosine kinase activity and therefore antibody-induced internalization of viral glycoproteins (Favoreel *et al.*, 1999). After complement incubation, cells were washed again in RPMI-1640® and finally resuspended in 0.5 ml RPMI-1640® supplemented with 10 µg propidium iodide (PI) (Molecular Probes, Eugene, Oregon, USA), which specifically stains dead cells. Fluorescence intensity of the cells was analyzed by flow cytometry after 5 min incubation with PI.

To determine the percentage of infected cells lysed by ADCML, the following formula was used:

$$\frac{\%\text{ of dead cells after the ADCML assay - \% of dead cells before the ADCML assay}}{\%\text{ of infected cells}} \times 100$$

Enrichment of infected cells with antibody-induced internalization of viral cell surface proteins by magnetic cell sorting. PRV-infected monocytes (13 h p.i.) were incubated for 1 h at 37°C with 0.9 mg/ml of FITC-labeled PRV-specific antibodies (without genistein). Cells were washed with phosphate buffered saline (PBS), supernatant was removed completely and the cells were resuspended in 90 µl of buffer, consisting of PBS at pH 7.2 supplemented with 0.5% bovine serum albumin and 2 mM EDTA. After washing, 10 µl of MACS (magnetic antibodies cell separation) anti-FITC Microbeads® (Miltenyi Biotec, Bergisch Gladbach, Germany) were added and cells were incubated for 30 min at 4°C. Cells were washed, resuspended in 500 µl buffer and sorted with the MiniMACS (Miltenyi Biotec), according to the manufacturer's instructions. The flow-through fraction, enriched in cells with complete internalization, was collected, washed in RPMI-1640® and used in the ADCML assay.

Definition of the viral protein distribution. The distribution of viral glycoproteins was scored as described before (Favoreel *et al.,* 1999; Van de Walle *et al.*, 2001): (i) "no internalization", when the fluorescence label exhibited a homogeneous or aggregated cell surface cover without internalized vesicles; (ii) "partial internalization", when the labeled viral glycoproteins formed randomly distributed aggregates on the cellular surface and some were localized in internalized vesicles; (iii) "complete internalization", when all visible viral glycoproteins were located in vesicles inside the cell, without any remaining on the plasma membrane. Quantitative results were obtained by examining the fluorescence distribution on at least 200 cells, by exciting with an Osram HBO 50-W bulb using a I3 filter and observing with a Leica DM IL fluorescence microscope (Leica, Germany). All assays were run independently at least three times.

Indirect immunofluorescence staining of viral cell surface proteins. PRV-infected monocytes (13 h p.i.) were centrifuged for 10 min at 500xg, washed and fixed for 10 min in 1% paraformaldehyde. The cells were washed and incubated for 1 h at 37°C with 0.9 mg/ml of porcine PRV-specific antibodies (originating from different hyperimmune sera as described in the text), followed by 1/300 FITC-labeled

rabbit α-swine antibodies (Nordic Immunological Laboratories, Tilburg, The Netherlands) for 1 h at 37°C. Finally, the cells were washed thoroughly and analyzed by flow cytometry.

PRV gE-gI IgG binding assay. PRV gE-gI IgG binding was assessed on PRV-infected swine kidney (SK)-cells. Therefore, seven days old confluent monolayers of SK-cells were trypsinized, inoculated with PRV Ka or PRV Ka gE-gI null mutant at a m.o.i. of 10 and further incubated in 25 cm^2 culture flasks for suspension culture (Sarstedt, Nümbrecht, Germany) on a tilting platform at 37°C in SK-medium, based on MEM® (Gibco BRL) and supplemented with 5% FBS, 0.3 mg/ml glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 0.1 mg/ml kanamycin. At 13 h p.i., cells were centrifuged for 10 min at 400xg, washed and resuspended in SK-medium. Cells were incubated for 1h at 37°C with 0.4 mg/ml of biotinylated PRV-negative porcine IgG antibodies (in the presence of 50 μg/ml genistein), essentially as described before (Favoreel *et al.*, 1997). To investigate the effect of different PRV-specific hyperimmune sera on the efficiency of gE-gI IgG binding, SK-cells (13 h p.i.) were first incubated with biotinylated PRV-negative IgG antibodies, as described above, followed by incubation for 1h at 37°C with 0.9 mg/ml IgG antibodies (in the presence of 50 μg/ml genistein) derived from hyperimmune sera of pigs inoculated with either PRV $Ka_{wild\text{-}type}$ or PRV $Ka_{gE\text{-}gI\ null}$, followed by streptavidin-FITC (in the presence of 50 μg/ml genistein) for 1h at 37°C and flow cytometric analysis.

Flow cytometric analysis. The intensity of fluorescence of the cells was analyzed with a Becton-Dickinson (San Jose, California) FACScalibur equipped with a 15-mW air-cooled argon ion laser and interfaced to a Macintosh Quadra 650 computer (Apple Computer, Inc., Cupertino, California) using BD Cellquest software. Acquisition rates were maintained at 100-200 cells/s. At least 5000 cells were analyzed for each sample and forward-scattered light versus side-scattered light dot plots were used to identify monocyte populations. Statistical analysis was performed with SPSS (SPSS Inc., Chicago, Illinois, USA).

Results

Effect of antibody and complement concentration on ADCML of PRV-infected monocytes. To optimize the ADCML assay, PRV-infected (Ka, Be, 89V87 or NIA3) porcine blood monocytes (13 h p.i.) were incubated with different concentrations (0-1.8 mg/ml) of PRV-specific antibodies or different concentrations (0 - 10%) of porcine complement. Genistein (50 μg/ml) was used to avoid internalization and had no effect on cell viability, as assessed by the use of propidium iodide (PI) and flow cytometric analysis. ADCML was shown to be specific, since (i) incubating mock-infected cells with 0.9 mg/ml PRV-specific IgG and subsequently with 5% porcine complement resulted in background levels of lysis (3.8 ± 2.5% dead cells) and (ii) incubating PRV Ka-infected cells with 0.9 mg/ml IgG antibodies derived from a PRV-negative pig and subsequently with 5% porcine complement also resulted in background levels of lysis (0.9 ± 1.6% dead cells).

Fig. 1.A&B shows that ADCML of PRV-infected monocytes increases with increasing concentrations of antibodies and complement. A steady-state level of cell lysis was observed when using at least 0.9 mg/ml of antibodies and 5% of porcine complement. These concentrations were therefore used in all further experiments. A low percentage (2.1% - 9.7%) of dead cells was observed when adding 5% of porcine complement without the addition of antibodies. To further investigate if this percentage could be attributed to the activation of the alternative complement pathway, experiments with the chelators EGTA and EDTA were performed. The Ca^{2+} and Mg^{2+}-chelator EDTA blocks the activation of both the classical and the alternative pathway and was used to examine if the observed cell death during ADCML is indeed complement-mediated (Joseph *et al.*, 1975; Friedman *et al.*, 2000). The Ca^{2+}-chelator EGTA only blocks the activation of the classical pathway and was used to determine if the low percentage of cell lysis observed when no PRV-specific antibodies were added in the ADCML assay, is due to activation of the alternative complement pathway (Joseph *et al.*, 1975; Friedman *et al.*, 2000). PRV Ka-infected cells were incubated with 10mM EDTA or 10mM EGTA (in combination with 1mM Mg^{2+}) 15 min before and during complement incubation. Adding 10mM EDTA (inhibitor of both classical and alternative pathway) during the ADCML assay

reduced the percentage of dead cells from 49.0% ± 3.6 to 1.3% ± 1.5 indicating that the observed lysis during ADCML is indeed complement-mediated. Adding 10mM EGTA (inhibitor of the activation of the classical pathway), in combination with 1 mM Mg^{2+}, resulted in a reduction of the percentage of lysed cells to 9.0% ± 1.0. This percentage is comparable to the percentage of cell lysis we observe when only complement and no antibodies are added during the ADCML assay (7.3% ± 0.6). Hence, we can conclude that the low percentage of dead cells when adding porcine complement without the addition of antibodies is most probably due to activation of the antibody-independent alternative complement pathway.

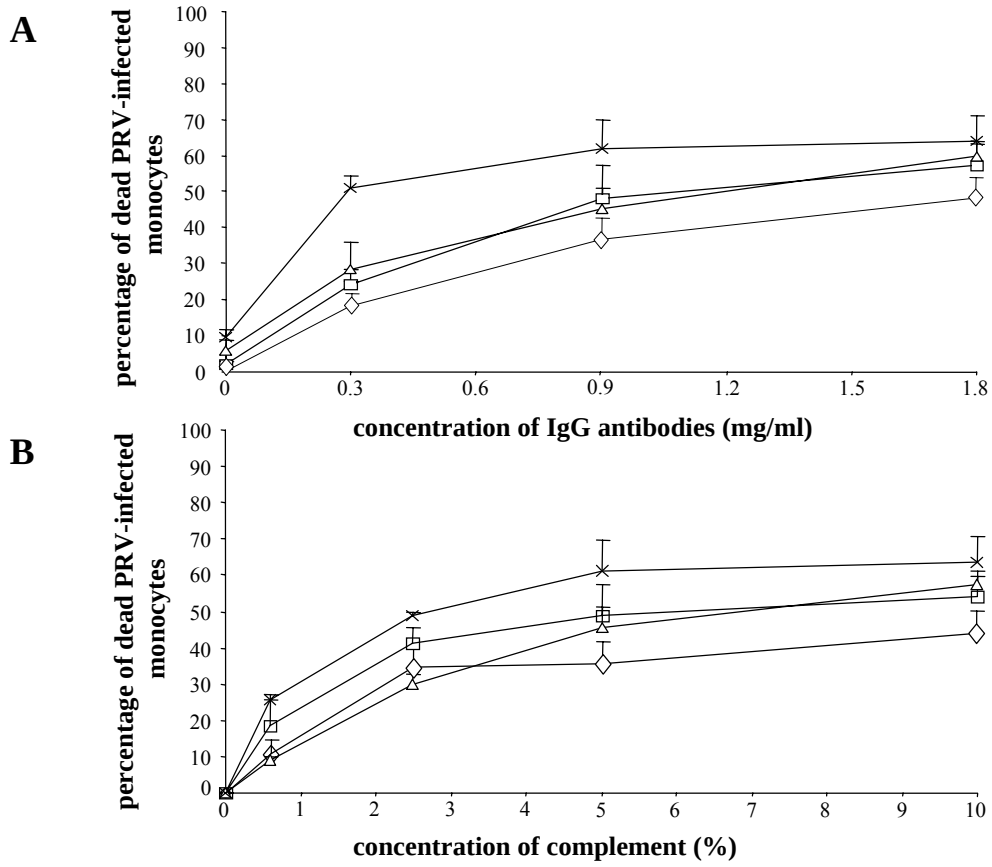

FIG. 1. (A)&(B). Effect of different concentrations of antibodies and porcine complement on ADCML of PRV-infected monocytes. Monocytes, at 13 h p.i. with Kaplan (×), 89V87 (Δ), Becker (◊) or NIA3 (□), were incubated for 1 h at 37°C with different concentrations of PRV-specific antibodies followed by 5% of porcine complement for 1 h at 37°C (A) or 0.9 mg/ml PRV- specific antibodies for 1 h at 37°C followed by different concentrations of porcine complement for 1 h at 37°C (B). Afterwards, PI was added for 5 min and percentage of dead PRV-infected monocytes was analyzed by flow cytometry. The data are means ± standard deviations of triplicate assays.

Further, we tested whether the use of different virus strains and IgG purified from different hyperimmune sera, resulted in different ADCML efficiencies. The use of

comparable concentrations of IgG antibodies derived from different porcine hyperimmune sera had no significant effect on the efficiency of ADCML nor did the use of three different PRV wild type strains result in significantly different susceptibilities of the infected monocytes toward ADCML (Fig. 2.). There was no increase in ADCML efficiency when incubating the cells with porcine complement for 2 or 3 h compared to 1 h (data not shown).

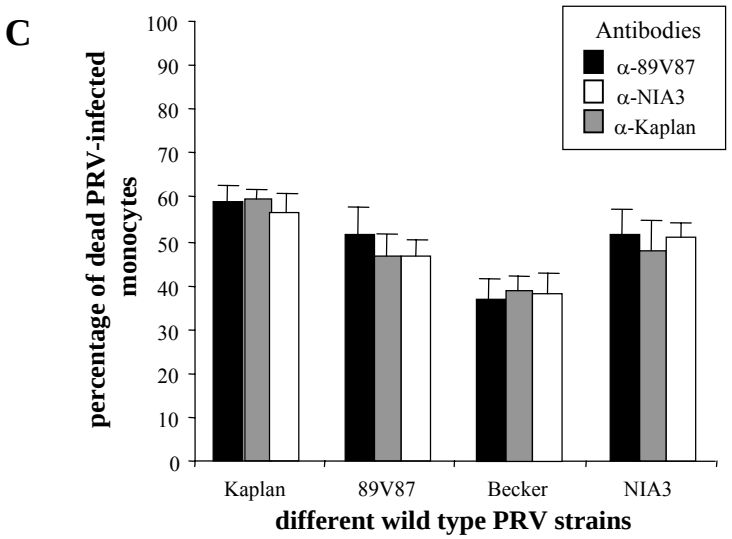

FIG. 2. Effect of antibodies derived from different hyperimmune sera on ADCML efficiency of PRV-infected monocytes. Monocytes, at 13 h p.i. with Kaplan, 89V87, Becker or NIA3, were incubated for 1 h at 37°C with antibodies derived from hyperimmune sera against 89V87 (black bars), NIA3 (grey bars) or Kaplan (white bars). Afterwards, cells were incubated with 5% of porcine complement for 1 h at 37°C. Finally, PI was added for 5 min and percentage of dead PRV-infected monocytes was analyzed by flow cytometry. The data are means ± standard deviations of triplicate assays.

Effect of antibody-induced internalization of viral cell surface glycoproteins on ADCML of PRV-infected monocytes. Addition of PRV-specific polyclonal antibodies to PRV-infected monocytes induces internalization of the majority of plasma membrane-anchored viral glycoproteins (Favoreel *et al.*, 1999). In order to investigate whether this process may interfere with ADCML of PRV-infected monocytes, PRV 89V87-infected monocytes (13 h p.i.) were incubated for 1 h at 37°C with 0.9 mg/ml FITC-labeled antibodies in the presence or absence of 50μg/ml genistein and subsequently used in the ADCML assay. Genistein is a tyrosine kinase inhibitor which blocks the internalization process (Favoreel *et al.*, 1999). The percentage of cells lysed by ADCML when internalization could occur (39.0% ± 2.0) was lower than the percentage of lysis when no internalization could occur (46.0% ±

3.5). Using a PRV strain with a point mutation in the tyrosine residue in the cytoplasmic tail of gB that is critical for efficient antibody-induced internalization (Favoreel *et al.*, 2002) resulted in a similar increase in ADCML of PRV-infected monocytes (50.7 ± 1.6% cell lysis versus 41.4 ± 3.4% when using the wild-type virus). Although these data together indicate that antibody-induced internalization is beneficial for the virus to avoid ADCML of infected cells, the differences in ADCML are relatively small. This can possibly be attributed to the fact that (in the absence of genistein and using a wild-type virus) only 36% of the cells had undergone complete internalization. To test this hypothesis, the population of infected cells with complete internalization was enriched by magnetic cell sorting (cfr. Materials and Methods). This enrichment had no effect on the viability of the cells (as assessed by using PI and flow cytometric analysis) and resulted in an increase of cells with internalized viral glycoproteins in the flow through fraction from 36.0 ± 6.2% to 74.3 ± 2.1% (Fig. 3.A). Of the latter population of cells, only 21% ± 6.6 were lysed by ADCML (Fig. 3. B), which is significantly less than the 46% ± 3.5 that were lysed by ADCML when no internalization could occur ($p < 0.005$, two way ANOVA) (Fig. 3.B).
Taking the results of the experiments with and without genistein and with and without magnetic cell sorting together, we calculated that approximately 47% of the cells with no or partial internalization are lysed by complement, whereas only about 12% of the cells with complete internalization are lysed by complement. The reasoning behind this calculation can be found as an addendum at the end of the Results section, page 45.

Effect of gE-gI on ADCML of PRV-infected monocytes. HSV gE-gI possesses Fc receptor activity, which has been shown to be important for immune evasion of the virus *in vivo* (Nagashunmugam *et al.*, 1998). Fc receptor activity has been demonstrated for PRV gE-gI as well, but its effect on immune evasion had not been investigated thus far (Favoreel *et* al., 1997). To examine the role of PRV gE-gI in ADCML evasion *in vitro*, $PRV_{wild\ type-}$ and $PRV_{gE\text{-}gI\ null}$-infected monocytes were used in the ADCML assay. First, an indirect immunofluorescence assay (cfr. Materials and Methods) was used to ensure that cells infected with the gE-gI mutants (13 h p.i.) did not bind lower amounts of PRV-specific antibodies, compared to cells infected with the wild type strains (Fig. 4. IA). Second, cells infected with the wild type strains or

gE-gI null strains (13 h p.i.) were subjected to the ADCML assay, using the same antibodies.

A

	% of PRV-infected cells with …… internalization		
	no	partial	complete
absence of genistein:			
before magnetic cell sorting	48.0 ± 5.2	16.0 ± 1.5	36.0 ± 6.2
after magnetic cell sorting			
flow through fraction	15.0 ± 1.0	10.7 ± 1.2	74.3 ± 2.1
retained fraction	82.4 ± 2.5	9.3 ± 1.2	8.3 ± 2.1
presence of genistein:	93.4 ± 1.2	3.0 ± 0.0	3.6 ± 1.1

B

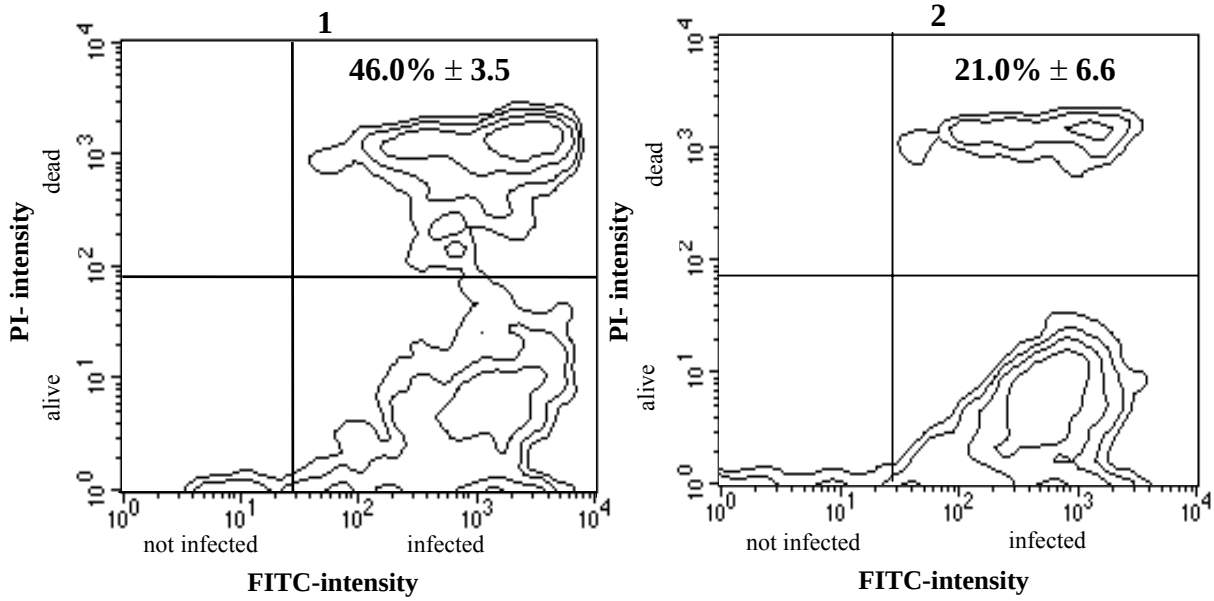

FIG. 3. Effect of antibody-induced internalization of viral cell surface proteins on ADCML of PRV-infected monocytes. PRV-infected monocytes (13 h p.i.) were incubated with FITC-labeled PRV-specific antibodies for 1 h at 37°C in the presence or absence of genistein (internalization inhibitor). Afterwards, cells with internalized viral proteins were enriched by magnetic cell sorting. (A) Percentage of cells with no, partial, or complete internalization in the absence of genistein (before and after enrichment) or in the presence of genistein. The data are means ± standard deviations of triplicate assays. (B) Representative flow cytometric contour plots showing the percentage of dead (PI-positive) infected (FITC-positive) monocytes after ADCML of monocytes without (+ genistein) (1) or with internalized viral protein-antibody complexes (- genistein, after enrichment by magnetic cell sorting) (2). Percentages given are means ± standard deviations of triplicate assays.

Fig. 4. IB shows that cells infected with the wild type strains were only slightly, but significantly, less susceptible ($p< 0.01$) to ADCML compared to cells infected with the gE-gI null mutants.

A possible explanation for the rather small difference in ADCML susceptibility between $PRV_{wild\ type}$- and $PRV_{gE\text{-}gI\ null}$-infected monocytes could be that gE-gI-specific antibodies that are present in the hyperimmune serum used in the ADCML assay, interfere with the PRV gE-gI Fc receptor activity. Therefore, the same ADCML experiments were repeated using antibodies derived from a hyperimmune serum of a pig infected with the $Ka_{gE\text{-}gI\ null}$ mutant and therefore does not contain gE-gI specific antibodies. Fig. 4. IIB shows that this resulted in a much more prominent difference in ADCML susceptibility between $PRV_{wild\ type}$- and $PRV_{gE\text{-}gI\ null}$-infected monocytes ($p<0.001$).

To ensure that the observed difference in ADCML susceptibility of wild type- or gE-gI null-infected cells, using the antibodies derived from gE-gI-negative hyperimmune serum, were not due to differences in the quantity of antibodies bound to the wild type- or gE-gI null-infected cells, an indirect immunofluorescence assay using the same antibodies was performed (Fig. 4. IIA). From the results of the ADCML assay, using wild type and gE-gI-negative hyperimmune sera and $PRV_{wild\ type}$ - and $PRV_{gE\text{-}gI\ null}$ -infected monocytes, it can be concluded (i) that the PRV gE-gI IgG binding, like HSV gE-gI IgG binding, protects infected cells from efficient ADCML *in vitro* and (ii) that there are indications that gE-gI specific antibodies can interfere with the PRV gE-gI Fc receptor activity.

To further investigate whether gE-gI-specific antibodies can interfere with PRV gE-gI Fc receptor activity, we performed a displacement assay on PRV Ka-infected swine kidney (SK)-cells. SK-cells, in contrast to monocytes, do not express natural Fc receptors on their cell surface and are therefore better suited to study the PRV gE-gI Fc receptor activity. The displacement assay consisted of preincubation of PRV-infected SK-cells with PRV-negative IgG, followed by incubation of the cells with PRV-specific IgG derived from hyperimmune sera of pigs inoculated with wild type PRV or gE-gI null PRV. If there are antibodies present among the PRV-specific antibodies that are directed against epitopes on gE that are important for Fc receptor activity, it is to be expected that they will be able to displace the PRV-negative IgG which were previously bound with their Fc side, since the affinity of IgG antibodies to

their antigen is typically higher than that of the Fc sides of antibodies to the Fc receptor (Fauger *et al.*, 1998; Friget *et al.*, 1998).

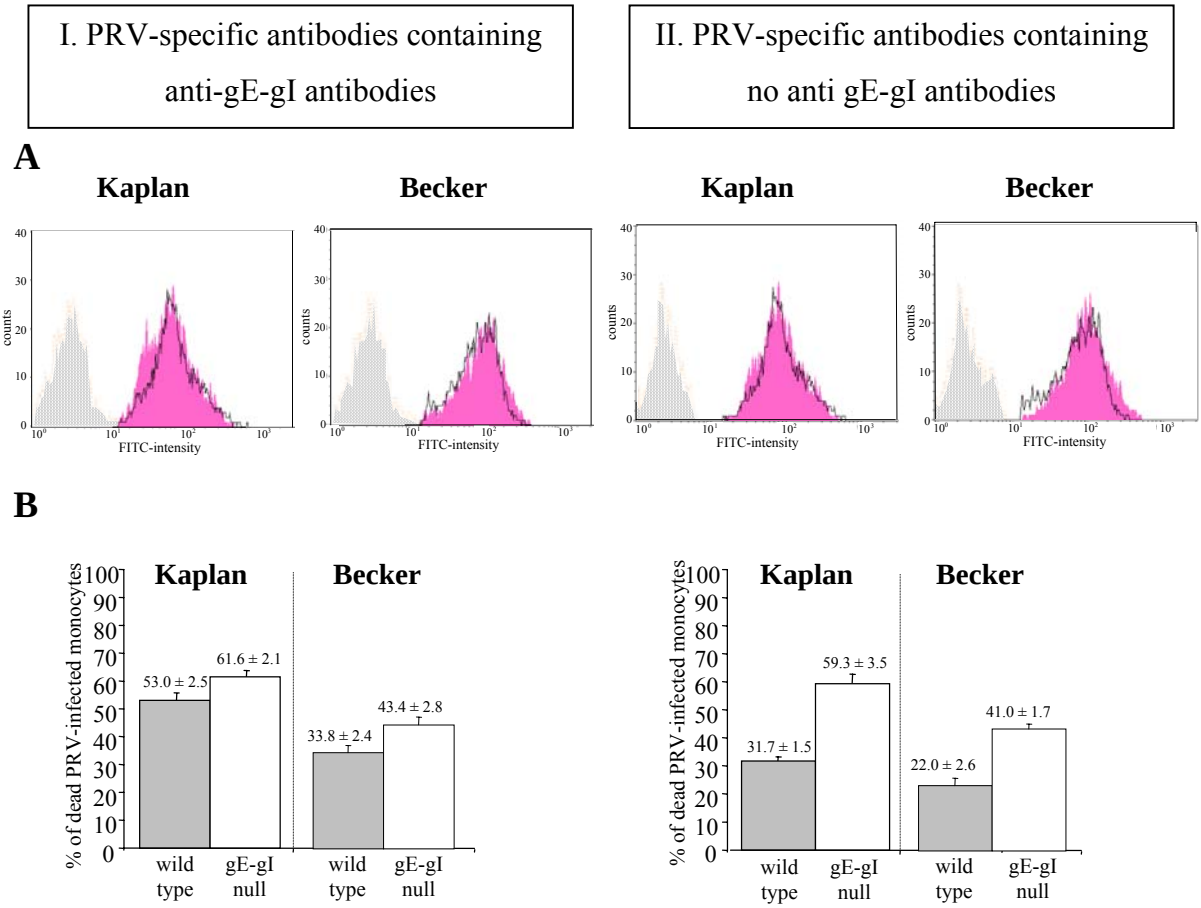

FIG. 4. Effect of gE-gI deletion on ADCML of PRV-infected monocytes using polyclonal PRV-specific antibodies containing gE-gI-specific antibodies (I) or containing no gE-gI-specific antibodies (II). (A) Fluorescence intensity of monocytes, 13 h p.i. with PRV wild type strains (grey shaded) or with the gE-gI null mutants (—) and stained using 0.9 mg/ml porcine PRV-specific antibodies containing gE-gI-specific antibodies (I) or containing no gE-gI-specific antibodies (II) for 1h at 37°C, followed by incubation for 1h at 37°C with FITC-labeled rabbit α-swine antibodies. Mock-infected monocytes were used as a negative control (dotted plots). Fluorescence intensity was analyzed by flow cytometry. (B) Effect of deletion of gE-gI on ADCML of PRV-infected monocytes when using PRV-specific antibodies containing gE-gI-specific antibodies (I) or containing no gE-gI-specific antibodies (II). Monocytes, at 13 h p.i. with the wild type strains (grey bars) or the gE-gI null mutants (white bars) were used in the ADCML assay. Afterwards, PI was added for 5 min and percentage of dead PRV-infected monocytes was analyzed by flow cytometry. The data are means ± standard deviations of triplicate assays.

Fig. 5. A, shows that wild type PRV-infected SK-cells exhibit IgG binding capacity, whereas gE-gI null-infected SK-cells do not, as has been shown before (Favoreel *et al.*, 1997). This was determined using a FACS assay as described in the Methods section. Fig. 5. B shows the results of the displacement assay. Here, $PRV_{wild\ type}$-infected cells were incubated with 0.4 mg/ml biotinylated PRV-negative IgG and subsequently with 0.9 mg IgG/ml derived from either wild type or gE-gI-negative

hyperimmune sera, followed by incubation with streptavidin-FITC and flow cytometric analysis of the fluorescence intensity. Antibodies, derived from pigs inoculated with wild type PRV were able to efficiently displace the biotinylated PRV-negative IgG, whereas antibodies derived from gE-gI null-infected pigs were not, supporting our hypothesis that gE-gI-specific antibodies can at least partially interfere with PRV gE-gI Fc receptor activity.

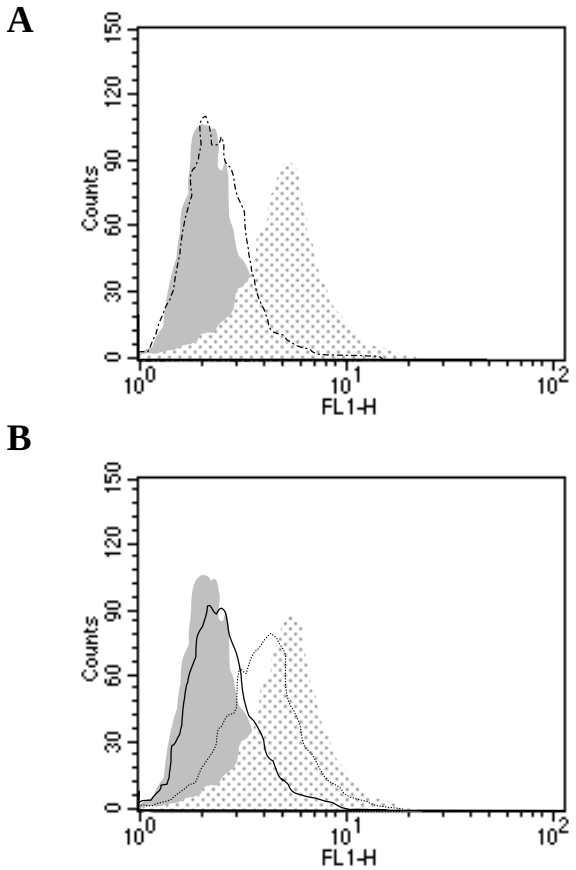

FIG. 5. (A). *PRV gE-gI Fc receptor activity*. Flow cytometric histogram showing fluorescence intensity of SK-cells, 13 h p.i. with PRV $Ka_{wild\ type}$ or with $Ka_{gE\text{-}gI\ null}$, incubated for 1 h at 37°C with 0.4 mg/ml biotinylated PRV-negative IgG, followed by incubation for 1 h at 37°C with streptavidin-FITC. PRV $Ka_{wild\ type}$ shows gE-gI Fc receptor activity (dotted plot), whereas PRV $Ka_{gE\text{-}gI\ null}$ does not (grey shaded). As a negative control, PRV $Ka_{wild\ type}$ –infected SK-cells were incubated with streptavidin-FITC only (dash/dotted line). (B). *Displacement assay*. The dotted plot and grey shaded plot represent the fluorescence intensity of the SK-cells, treated as described in (A). PRV $Ka_{wild\ type}$ –infected cells were incubated for 1h at 37°C with 0.4 mg/ml of biotinylated porcine PRV-negative IgG, followed by incubation for 1 h at 37°C with 0.9 mg/ml of PRV-specific IgG antibodies derived from hyperimmune sera of pigs infected with either wild type PRV or gE-gI null PRV. Finally, SK-cells were incubated for 1 h at 37°C with streptavidin-FITC. Bound PRV-negative IgG were efficiently displaced by antibodies derived from hyperimmune serum of a pig infected with wild type PRV (solid line) but not when using antibodies derived from hyperimmune serum of a pig infected with gE-gI null PRV (dotted line).

ADDENDUM: Calculation of ADCML susceptibility for cells with and without internalization

We calculated the susceptibility towards cells with complete internalization (12%) and cells without internalization (47%) as follows:
We based the calculations on the percentage of flow cytometrically determined complement-mediated lysis of two mixed populations: one population with a predominant number of cells without complete internalization (in the presence of genistein) and one population with a predominant number of cells with complete internalization (absence of genistein, enrichment of cells with complete internalization by magnetic cell sorting). These calculations were done as follows:

Sensitivity of cells without complete internalization towards complement-mediated lysis = x
Sensitivity of cells with complete internalization towards complement-mediated lysis = y

Equation 1:
In the presence of genistein: 96% of the cells do not have complete internalization, 4% of the cells do have complete internalization. Of this total population of cells, 46% is lysed by complement (Fig.3a&b).
Hence: $96x + 4y = 46$

Equation 2:
In the absence of genistein, after enrichement of cells with complete internalization: 26% of the cells do not have complete internalization, 74% do have complete internalization. Of this total population of cells, 21% is lysed by complement (Fig.3a&b).
Hence: $26x + 74y=21$

To solve the equations:
Equation 1:
$96x + 4y = 46$

$$y = \frac{46 - 96x}{4}$$

Introduce this in equation 2:

$$26x + \frac{74(46 - 96x)}{4} = 21$$

resulting in $x = 0.47$, and introducing this in equation 1 (or 2) gives $y = 0.12$

Hence, the sensitivity of cells without complete internalization (x) is 0.47, so 47% of these cells are lysed by complement.
The sensitivity of cells with complete internalization (y) is 0.12, so only 12% of these cells are lysed by complement.

To check the reliability of these percentages, we compared them to the flow cytometrically determined percentages of complement-mediated lysis of a third population of cells (no genistein, no enrichment by magnetic cell sorting). Here we had 64% of cells without complete internalization, 36% of cells with complete internalization, and this resulted in 39% of the total population of cells that were lysed by complement (Fig.3a and page 39).
The equation would be:

$64x + 36y$

Introducing the calculated values for x and y (x=0.47, y=0.12) in the equation gives an estimated percentage of lysis of 34.4%, which is, although somewhat underestimated, fairly close to the actual percentage of lysis (39.0% ± 2.0%)(page 39). Hence, we concluded that the calculated percentages of sensitivity (12% and 47%) are fairly good estimates for the actual sensitivities of cells with or without complete internalization towards antibody-dependent complement-mediated lysis.

Discussion

PRV-infected blood monocytes are known to be able to transport PRV in well-vaccinated pigs (Wittmann *et al.*, 1980; Nauwynck & Pensaert, 1992). These infected monocytes express viral glycoproteins on their plasma membrane and should therefore be susceptible to antibody-dependent complement-mediated lysis (ADCML). Recently, a mechanism has been postulated of how PRV-infected monocytes potentially may avoid ADCML: the antibody-induced internalization of the majority of viral plasma membrane proteins (Favoreel *et al.*, 1999). The aim of the current study was therefore to evaluate the importance of this mechanism in the ADCML evasion of PRV-infected monocytes *in vitro*. Further, a second potential ADCML evasion strategy has been described for several alphaherpesviruses, including PRV: gE-gI Fc receptor activity. For HSV, the prototypical alphaherpesvirus, this gE-gI IgG binding has been shown to interfere with efficient antibody-dependent lysis *in vitro* and *in vivo* (Johnson *et al.*, 1988; Frank & Friedman, 1989; Nagashunmugam *et al.*, 1998). Here, we also investigated the effect of the PRV gE-gI Fc receptor activity on the efficiency of ADCML of PRV-infected monocytes *in vitro*. A third potential mechanism of complement evasion, gC complement binding, has also been described for several alphaherpesviruses, including HSV and PRV (Friedman *et al.*, 1984; Huemer *et al.*, 1993). For HSV, it has already been shown that this gC complement binding is important for evasion of the antibody-independent, alternative complement activation pathway, rather than for the antibody-dependent classical pathway (ADCML) (Friedman *et al.*, 1984; Friedman *et al.*; 1996; Lubinski *et al.*, 1999; Friedman *et al.*, 2000). Therefore, this mechanism was not considered in the current study.

An *in vitro* ADCML assay was set up, using PRV-infected porcine blood monocytes, porcine PRV-specific antibodies, and porcine complement, to simulate the *in vivo* situation. During the set up of this ADCML assay, we observed that a low percentage (up to 10% ± 2) of PRV-infected monocytes was lysed, when porcine complement was added to PRV-infected monocytes without prior addition of antibodies. This was not seen when using mock-infected cells and the low percentage of lysed cells was confirmed to be caused by activation of the alternative complement pathway, which does not require the presence of antibodies (reviewed by Pangburn &

Müller-Eberhard, 1984). Our results therefore suggest that although complement-mediated lysis of PRV-infected monocytes via the alternative pathway indeed does occur, it seems to be of rather minor importance. Efficient antibody-independent complement-mediated lysis of PRV-infected cells may perhaps be disturbed by binding of viral glycoprotein C to complement factor C3b (Huemer *et al.*, 1993). Activation of the alternative complement pathway by PRV-infected cells has already been described before (Kimman *et al.*, 1992). The percentage of infected cells lysed by the alternative complement activation in this study was approximately 20%, which is higher than the percentage observed in our study. The differences in cell type (swine kidney cells) and complement source (rabbit complement) can probably account for these differences.

Antibody-induced internalization of plasma membrane-bound viral glycoproteins in PRV-infected monocytes leaves the infected monocyte with only few viral protein-antibody complexes on its surface (Favoreel *et al.*, 1999). Since ADCML of infected cells principally depends on the density of antigen-antibody complexes on the cell surface (reviewed by Sissons & Oldstone, 1980), our hypothesis was that this internalization process may interfere with efficient ADCML. To investigate this, PRV-infected monocytes were incubated with PRV-specific antibodies in the absence or presence of genistein (a tyrosine kinase inhibitor that blocks the internalization (Favoreel *et al.*, 1999)), prior to incubation with complement. From these experiments, it could be concluded that cells with internalized viral glycoproteins are significantly less susceptible toward ADCML than cells that did not undergo internalization ($p < 0.005$): 47% of the cells without internalization of viral cell surface proteins were lysed by ADCML, compared to only 12% of the cells that had undergone internalization of viral cell surface glycoproteins. Since allowing antibody-induced internalization of antigen-antibody complexes to proceed protects PRV-infected monocytes from efficient ADCML *in vitro*, and since the internalization process in PRV-infected monocytes is fast and efficient, starting within minutes after antibody addition (Favoreel *et al.*, 1999), it is tempting to speculate that this process may be significant for the survival of infected monocytes in vaccinated animals. Further research will clarify whether the short time-span between antibody binding and internalization is short enough to allow a significant percentage of infected

monocytes to successfully avoid lysis by antibody-dependent components of the immune system.

For HSV, varicella-zoster virus (VZV) and PRV, the gE-gI complex has been demonstrated to display Fc receptor activity (Johnson *et al.*, 1988; Litwin *et al.*, 1992; Favoreel *et al.*, 1997). The expression of the gE-gI complex results in a species specific high affinity binding of IgG and it has been suggested that this Fc receptor activity may cause 'antibody bipolar bridging' resulting in disarmed bound antibodies which are unable to activate the classical complement pathway (Frank & Friedman, 1989). For HSV, gE-gI IgG binding has been shown to be important for immune evasion of the virus *in vivo* (Nagashunmugam *et al.*, 1998). In the current report, we show that, as expected, porcine blood monocytes infected with gE-gI-deleted PRV mutants are more susceptible to ADCML, compared to wild type-infected cells. This difference in percentage of lysed infected cells was significant ($p < 0.01$), but surprisingly low. However, the difference in ADCML between wild type and gE-gI null-infected monocytes became much more prominent when the ADCML assay was performed using antibodies derived from a hyperimmune serum of a pig infected with the $Ka_{gE\text{-}gI\ null}$ mutant. Additionally, we showed that antibodies derived from a hyperimmune serum of a pig, infected with wild type PRV, but not from a pig infected with the PRV gE-gI null mutant can at least partially interfere with efficient PRV gE-gI IgG binding. Taken all these findings together indicates that the PRV gE-gI Fc receptor activity (i) aids in protecting PRV-infected monocytes from efficient ADCML *in vitro* and (ii) that gE-gI-specific antibodies can at least partially interfere with efficient IgG binding by PRV gE-gI.

During all ADCML assays we performed, even when blocking internalization and using gE-gI null virus to avoid Fc binding of the antibodies, ADCML of infected cells never reached 100%. This may suggest that, besides the two mechanisms studied in this report, PRV may have other tools at its disposal to avoid ADCML of infected cells. Moreover, since a significant difference ($p < 0.05$) in the percentage of lysed infected cells was always observed when using either PRV strain Kaplan or Becker (Be-infected monocytes were always 1.5-fold less susceptible toward ADCML than Ka-infected monocytes), these other possible evasion mechanisms of PRV could be more effective for certain PRV strains than for others. One such mechanism could be PRV gC complement binding (Huemer *et al.*, 1993). The binding of gC to the C3 factor of the complement cascade was first described for HSV (Friedman *et al.*, 1984)

and has been shown to be important for immune evasion of HSV *in vivo* (Lubinski *et al.*, 1999). Although HSV gC has been shown to interfere only with the antibody-independent and not the antibody-dependent (ADCML) pathway of complement activation (Fries *et al.*, 1985; Friedman *et al.*, 1996), a possible role for this glycoprotein in protecting PRV-infected cells from ADCML cannot at present be ruled out and may be worth investigating in further detail. Several other mechanisms of ADCML evasion of infected cells have been described for other viruses and include the expression of viral proteins that mimic the function of cellular inhibitors of complement activation (Mold *et al.*, 1988; Albrecht & Fleckenstein, 1992; Isaacs *et al.*, 1992; Russo *et al.*, 1996; Spiller *et al.*, 1996; Saifuddin *et al.*, 1997; Kapadia *et al.*, 2000). Further research will be necessary to elucidate whether PRV makes use of such or other mechanisms to avoid ADCML of infected cells.

Furthermore, even when using a non-mutated virus and allowing antibody-induced internalization to proceed, a substantial number of PRV-infected cells (39.0 ± 2.0%) were lysed by ADCML. This most likely can be attributed to the fact that none of the complement evasion mechanisms is absolute. Antibody-induced internalization, which removes antigen-antibody complexes from the cell surface, only occurs completely in 36% of PRV-infected monocytes, leaving the other 64% unprotected. Also, the gE-gI Fc receptor activity may be partially inhibited to perform its function by the presence of gE-specific antibodies in serum (see Fig. 4). As discussed above, gC complement binding has been shown to be predominantly important for evading antibody-independent complement activation. These three arguments together possibly explain why a significant percentage of PRV-infected monocytes is sensitive towards ADCML. However, immune evasion strategies of PRV still allow a significant fraction of PRV-infected monocytes to avoid ADCML *in vitro*. This indicates that, although not perfect, PRV complement evasion strategies may still provide PRV-infected cells with an important tool to extend their life span and time to transmit virus throughout the body.

In conclusion, this report shows that antibody-induced internalization of PRV plasma membrane proteins, together with PRV gE-gI Fc receptor activity, are of significant importance in protecting PRV-infected monocytes from efficient ADCML *in vitro*.

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3.

INVOLVEMENT OF CELLULAR CYTOSKELETON COMPONENTS IN ANTIBODY-INDUCED INTERNALIZATION OF VIRAL GLYCOPROTEINS IN PSEUDORABIES VIRUS-INFECTED MONOCYTES

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Van de Walle, G. R., Favoreel, H. W., Nauwynck, H. J., Van Oostveldt, P. & Pensaert, M. B.

Summary

Pseudorabies virus (PRV) may still cause abortion, even in the presence of a vaccination-induced immunity and blood monocytes have been shown to be essential to transport the virus throughout the body of these immune animals. It has been demonstrated recently that addition of anti-PRV polyclonal antibodies to PRV-infected monocytes induces internalization of plasma membrane-anchored viral proteins and this may interfere with antibody-dependent cell lysis. In the present study, it was shown that actin, microtubules, clathrin and dynein, the major cellular components involved in physiological endocytosis, are used by PRV to generate the antibody-induced internalization of viral glycoproteins. In a first assay, porcine monocytes, inoculated with PRV for 13 hours, were treated with different concentrations of chemical inhibitors like colchicine, cytochalasin D, latrunculin B and amantadine-HCl, which inhibit polymerization of microtubules, actin/clathrin, actin and clathrin respectively, before the addition of PRV-specific antibodies. This resulted in a significant reduction of internalization compared to the non-treated control. In a second assay, double labeling experiments of viral proteins on the one hand and cellular components on the other hand were performed during the internalization process. A clear co-localization of actin, microtubules, clathrin and dynein with the viral proteins was observed at different stages during the internalization process. All these results indicate that cellular components are involved in the virological internalization process.

Introduction

Pseudorabies virus (PRV) is a member of the *Alphaherpesvirinae*, which causes Aujeszky's disease in its natural host, the pig. The clinical signs of the disease depend on the age of the pig and are characterised by nervous signs in newborn pigs, respiratory disorders in fattening pigs and reproductive failure in sows. Abortion may be an important consequence of PRV infection in susceptible pregnant sows (Pensaert & Kluge, 1989). In the presence of vaccination immunity, PRV inoculation can still result in infection of the respiratory tract, involving mononuclear cells in draining lymph nodes. These cells may enter the bloodstream, resulting in a restricted viraemia (Wittmann *et al*., 1980). The restricted replication in immune animals generally does not cause problems. However, abortion may occur as a result of cell-mediated transplacental spread and intrafetal replication. It has been shown that blood monocytes are essential to transport the virus in vaccination-immune pigs (Nauwynck & Pensaert, 1992; Nauwynck & Pensaert, 1995a). However, little is known on the exact mechanism how these PRV-infected monocytes survive in the blood without being eliminated by antibody-dependent cell lysis or cytotoxic T-lymphocytes.

Recently, it was demonstrated by Favoreel *et al.* (1999) that addition of PRV-specific antibodies to PRV-infected monocytes resulted in an aggregation of the membrane-bound viral proteins, followed by the internalization of these antigen-antibody complexes. This fast and efficient antibody-induced internalization process is mediated by the viral proteins gB and gD and may interfere with antibody-dependent cell lysis (Favoreel *et al.*, 1999).

Antibody-induced internalization of viral cell surface proteins resembles the well-studied bivalent ligand-induced endocytosis of cellular receptors (Favoreel *et al.*, 2000). It is known that binding of such a ligand to its receptor induces aggregation of the receptors. This results in a conformational change by which adaptor proteins (AP) can bind to certain highly conserved amino acid sequences in the cytoplasmic tail of the receptor. These AP will then bind to clathrin molecules, the driving force behind membrane invagination, with formation of clathrin-coated pits, which are finally pinched off into the cytoplasm as clathrin-coated vesicles (Goldstein *et al.*, 1985; Hirst & Robinson, 1998). It is assumed that actin plays an important role during this stage of endocytosis (reviewed by Qualmann *et al*., 2000). Clathrin-coated vesicles

will then deliver the receptor-ligand complexes to early endosomes, with the receptors directed back to the plasma membrane via a recycling pathway and the ligand proteins sorted into a lysosomal pathway (Ghosh *et al.,* 1994). The transport of the lysosomes to the centre of the cell is performed by the motor protein dynein, which mediates vesicle or organelle transport and moves along microtubules (reviewed by Goodson *et al.,* 1997; Hamm-Alvarez, 1998).

The main purpose of the current study was to investigate whether several components of the cellular cytoskeleton (clathrin, actin, dynein and microtubules), known to play a role in the process of bivalent ligand-induced endocytosis of cellular receptors, are being used in PRV-infected monocytes to generate the antibody-induced internalization of viral cell surface proteins.

Materials and Methods

Virus strains. PRV strains 89V87 and Kaplan (Ka) and PRV (Ka) deletion mutants gB^-, gC^-, gD^-, gE^-, gH^- and gM^- were used. All strains have been described earlier (Dijkstra *et al.*, 1996, Kaplan & Vater, 1959, Klupp *et al.*, 1992, Mettenleiter *et al.*, 1987, 1988; Nauwynck & Pensaert, 1992; Rauh & Mettenleiter, 1991). Strains carrying deletions in the genes encoding essential glycoproteins were grown on complementing cell lines.

Isolation of porcine blood monocytes. Pigs from a PRV-negative farm were used as blood donors. Blood was collected from the *vena jugularis* on heparin (15 U/ml) (Leo, Zaventem, Belgium). Blood mononuclear cells were separated on Ficoll Paque (Pharmacia Biotech AB, Uppsala, Sweden) following the manufacturer's instructions. Mononuclear cells were then resuspended in medium A, based on RPMI-1640 (Gibco BRL, Life Technologies Inc., Gaithersburg, MD) supplemented with 10% fetal calf serum (FCS), 0.3 mg/ml glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.1 mg/ml kanamycin, 1 mM sodium pyruvate, 1% non-essential amino acids 100x (Gibco BRL) and 10 U/ml heparin. Afterwards, cells were seeded on a 4-well chambered coverglass (Nunc A/S, Roskilde, Denmark) at a concentration of $2.5x10^6$ cells/ml and cultivated at 37°C with 5% CO_2. After 48 h, non-adherent cells, mainly consisting of lymphocytes, were removed by washing the chambered coverglass three times with RPMI-1640.

Inoculation of blood monocytes. The adherent cells, consisting of ≥70% of monocytes (as assessed by flow cytometric analysis after incubation with 74.22.15 (Pescovitz *et al.*, 1984)), were inoculated with different PRV strains at a m.o.i. of 20 in 0.5 ml medium A without heparin. Monocytes were incubated for 13 h at 37°C with 5% CO_2.

Incubation of PRV-infected monocytes with porcine anti-PRV polyclonal antibodies. Monocytes, 13 h after inoculation with PRV, were washed three times with RPMI-1640 and incubated for 1 h at 37°C with FITC-conjugated PRV-specific polyclonal antibodies (0.33 mg/ml) (as described by Favoreel *et al.,* 1999). These

FITC-labeled, protein A-purified IgG antibodies were, as described earlier (Nauwynck & Pensaert, 1995b), derived from a PRV (89V87)-inoculated pig originating from a PRV-negative farm and had a titer of 512, as determined with a serum neutralization (SN) test (Andries *et al.*, 1978). At different time points (0, 2, 5, 10, 30 and 60 min) post antibody addition (p.Ab.a), cells were fixed with 0.4% formaldehyde (except where indicated otherwise). Finally, the cells were washed thoroughly, mounted in a glycerin-phosphate buffered saline solution (PBS) (0.9:0.1, vol/vol) with 2.5% 1,4-diazabicyclo(2.2.2)octane (DABCO; Janssen Chimica, Beerse, Belgium), excited with an Osram HBO 50-W bulb using a I3 filter, and observed with a Leica DM IL inverted fluorescence microscope (Leica, Germany).

Definition of the viral glycoprotein distribution. The viral cell surface protein distribution was scored as a 'rim' when the fluorescence label exhibited a homogeneous cell surface cover. The cells were scored as 'patched' when the labeled viral proteins formed randomly distributed aggregates on the cellular surface. The viral proteins were considered 'internalized' when all visible viral proteins were located in vesicles inside the cell, without any remaining on the plasma membrane. Quantitative results were obtained by examining the fluorescence distribution on at least 200 cells. All assays were run independently at least three times.

Chemical inhibitors of the different cellular components. To determine the involvement of the cellular components during the observed process of antibody-induced internalization, different concentrations of colchicine (Sigma-Aldrich Chemie GmbH, Steinheim, Germany; inhibitor of tubulin polymerization; 0 μM-500 μM), cytochalasin D (Sigma-Aldrich Chemie GmbH, Steinheim, Germany; inhibitor of G-actin polymerization to F-actin and inhibitor of budding of clathrin-coated vesicles; 0 μM-100 μM), latrunculin B (ICN Biochemicals Inc., Ohio; actin-disrupting agent; 0 μM-50 μM) and amantadine-HCl (Sigma-Aldrich Chemie GmbH, Steinheim, Germany; inhibitor of clathrin-coated-pit invagination at the plasma membrane; 0 μm-500 μM) were added 30 min before and during antibody incubation. For each concentration of the chemical inhibitors, the viability of the monocytes was analyzed by flow cytometry after the addition of 10 μg propidium iodide (Molecular Probes, Eugene, Oregon). Only concentrations with no significant effect on cell viability were

used. Statistical analysis was performed with SPSS (SPSS Inc., Chicago, Illinois, USA).

Double immunofluorescence labeling of viral proteins with microtubules or actin. After incubation with FITC-conjugated PRV-specific antibodies, as described above, the cells were washed in a cytoskeleton-stabilizing buffer (CSB), containing 10 mM Pipes buffer, 150 mM NaCl, 5 mM EGTA, 5 mM MgCl and 5 mM glucose and afterwards fixed for 10 min at 37°C with a 3% solution of paraformaldehyde in CSB. After washing in CSB, cells were permeabilized for 2 min in a 0.1% solution of Triton-X 100 in CSB and then washed again in CSB.

Microtubules were stained by incubation of cells for 1 h at 37°C with mouse anti-α-tubulin IgG1 antibodies 1:20 in CSB (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Afterwards, cells were washed in washing buffer (WB), containing 200 mM Tris, 1.45 M NaCl, 20 mM EGTA and 20 mM MgCl and subsequently incubated for 1 h at 37°C with goat-anti-mouse IgG-Texas Red 1:40 in CSB (Molecular Probes, Eugene, Oregon) and washed twice in WB.

To stain actin, cells were incubated for 1 h at 37°C with 200 nM phalloidin-Texas Red (Molecular Probes, Eugene, Oregon) in CSB and afterwards washed twice in WB.

Double immunofluorescence labeling of viral proteins with clathrin or dynein. After incubation with FITC-conjugated PRV-specific antibodies, as described above, the cells were washed with Tris-buffered saline (containing 20 mM Tris-HCl and 150 mM NaCl) + 4.5% sucrose (w/v) + 2% heat-inactivated goat serum (TBS-SG), fixed in a 3% solution of paraformaldehyde and permeabilized with methanol 100% as described by Racoosin & Swanson (1994).

Clathrin was stained by incubation of cells for 1 h at 37°C with mouse anti-clathrin IgM antibodies (ICN Biomedicals Inc., Ohio) 1:100 in PBS, supplemented with 0.3% gelatine, and dynein by incubation with mouse anti-dynein IgM antibodies (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) 1:100 in PBS, supplemented with 0.3% gelatine. Afterwards, cells were washed in TBS-SG and incubated for 1 h at 37°C with rat-anti-mouse IgM-biotin (Serotec Ltd, Oxford, UK) 1:250 in PBS, supplemented with 0.3% gelatine. After washing, cells were incubated for 1 h at 37°C

with streptavidin-Texas Red 1:100 in PBS, supplemented with 0.3% gelatine, (Molecular Probes, Eugene, Oregon) and washed twice in TBS-SG.

Confocal Laser Scanning Microscopy. Fluorescent double stained samples were examined on a Bio-Rad Radiance 2000 confocal laser scanning system (Bio-Rad House, Hertfordshire, UK), linked to a Nikon Diaphot 300 microscope (Nikon Corporation, Tokyo, Japan) and interfaced to an AST Premmia SE 4/66d computer (AST Computer, Irvine, California). Krypton/Argon laser light was used to excite FITC (488 nm line) and Texas Red (568 nm line) fluorochromes using a K1- and a K2-emission filter respectively. Extended focus images were obtained with Bio-Rad COMOS Software. Images were printed on a Kodak XLS 8600 PS printer (Eastman Kodak Company, Rochester, New York).

Results

Kinetics of antibody-induced internalization of PRV-infected monocytes in adhesion versus suspension and the importance of viral proteins gB and gD. In the study of Favoreel et al. (1999), monocytes in suspension were used to investigate the kinetics of the antibody-induced redistribution and internalization of viral cell surface proteins, expressed on PRV-infected monocytes. To obtain better confocal images, monocytes in adhesion were used in this study. Identical experiments as described by Favoreel et al. (1999) were performed first, to evaluate whether monocytes in adhesion behave like monocytes in suspension. The evolution of redistribution of the viral glycoproteins after addition of PRV-specific antibodies did not differ between PRV-infected monocytes in adhesion and monocytes in suspension (Fig. 1A). Shortly after antibody addition, viral proteins became aggregated in patches and then rapidly internalized. The only visible difference between monocytes in adhesion and monocytes in suspension was that the internalization process was more efficient when monocytes were in adhesion. After 1 h of incubation with PRV-specific IgG, the percentage of cells that showed internalized viral cell surface proteins was 85% in adhesion, which is higher than the value previously obtained by Favoreel et al. (1999) with monocytes in suspension (65%).

In the study of Favoreel et al. (1999) it has been shown, by the use of PRV (Ka) and isogenic deletion mutants, that the viral proteins gB and gD are very important for the viral protein internalization process in suspension. Similar experiments with PRV (Ka) and the gB and gD deletion mutant strains were performed with monocytes in adhesion. From Fig. 1B it can be concluded that viral proteins gB and gD are also of significant importance in the internalization process for monocytes in adhesion ($P<0.001$, one way anova). As an extra control, experiments using the PRV (Ka) deletion mutants gC, gE, gH and gM were performed and none of these mutants had an impaired ability to induce internalization, which is also consistent with previously described results with monocytes in suspension (Favoreel et al., 1999) (data not shown). Therefore, in all further experiments adherent monocytes were used.

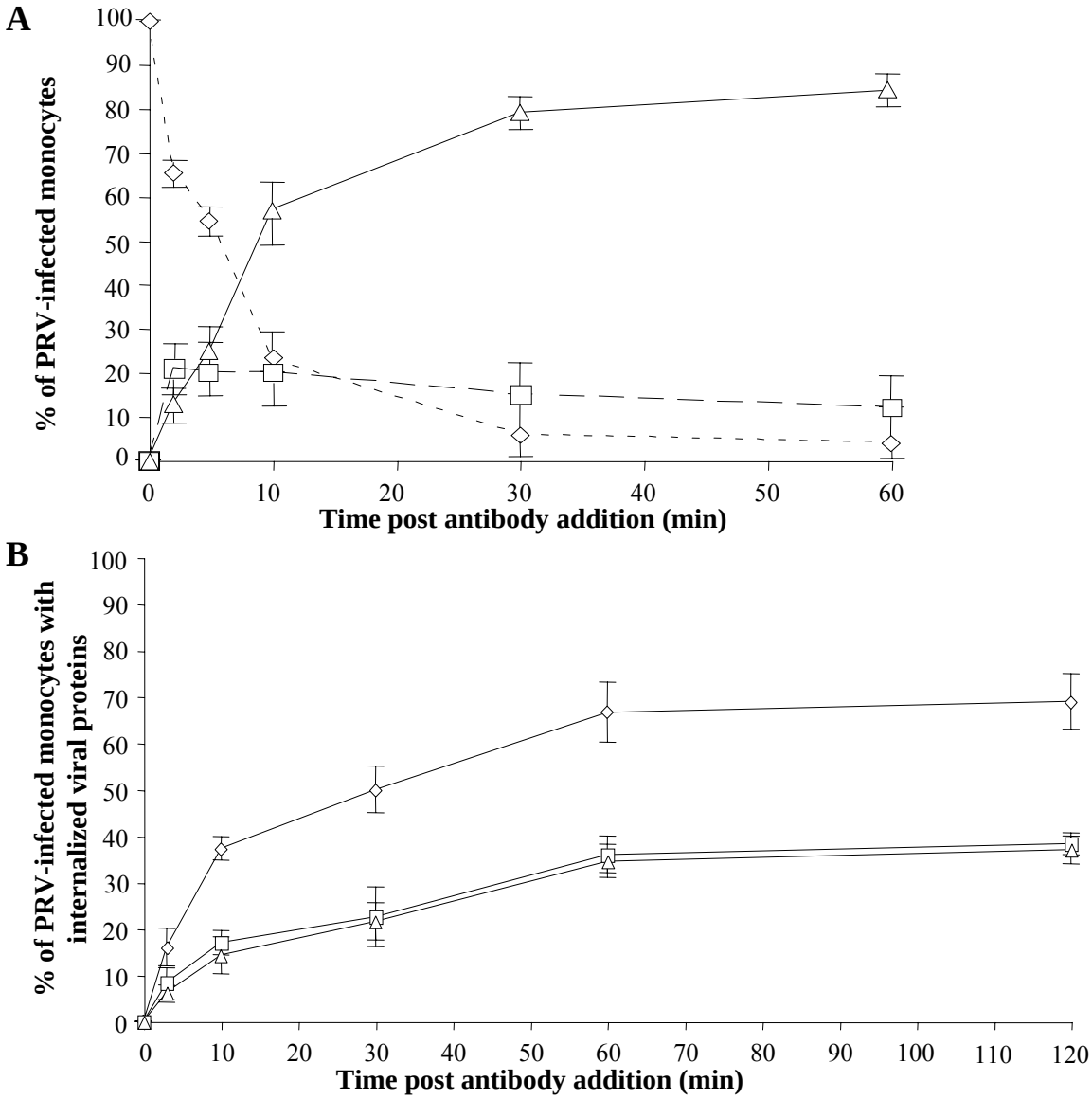

FIG. 1. (A). *Antibody-induced redistribution of viral glycoproteins expressed on the plasma membrane of PRV-infected monocytes in adhesion.* PRV-infected monocytes were incubated at 37°C with FITC-labeled PRV-specific antibodies and fixed at different time points during this incubation. Distribution of viral glycoproteins was observed by fluorescence microscopy and scored as described in Materials and Methods. Curves indicate percentages of PRV-positive cells with rims (◊), patches (🕯) and internalized viral cell surface proteins (Δ). The data are means ± standard deviations of triplicate assays.

(B). *Viral glycoprotein internalization is mediated by viral proteins gB and gD.* Monocytes in adhesion were infected with PRV (Ka) and gB and gD deletion mutant strains. PRV-infected monocytes were incubated at 37°C with FITC-labeled PRV-specific antibodies and fixed at different time points during this incubation. Curves indicate percentages of monocytes with internalized viral cell surface proteins when infected with PRV (Ka) (◊), gB deletion mutant (🕯) and gD deletion mutant (Δ) strains. The data are means ± standard deviations of triplicate assays.

Influence of chemical cellular inhibitors on antibody-induced internalization of viral cell surface proteins. During different steps of the well-studied process of ligand-induced endocytosis of cellular receptors, microtubules, actin, clathrin and dynein are of critical importance (Goldstein et al., 1985; Hirst & Robinson, 1998; Merrifield et al., 1999). Experiments with different chemical inhibitors were performed to investigate whether these components of the cellular cytoskeleton are of importance in the process of internalization of viral cell surface proteins. The concentrations of colchicine, cytochalasin D and amantadine-HCl are similar as those used in several other studies (Bourguignon & Bourguignon, 1984; Cooper, 1987; Everitt & Rodriguez, 1999; Favoreel et al., 1997). For latrunculin B, an actin-disrupting agent, a concentration of 50 μM was used, based on the effect of different concentrations of the agent on the monocyte actin filaments (determined via phalloidin-Texas Red staining) and on cell viability (determined via propidium iodide staining). None of the reagents caused significant decreases in the expression of viral glycoproteins on the plasma membrane, nor did they affect patching of viral cell surface proteins after the addition of antibodies, as analyzed by fluorescence microscopy (data not shown). All the different concentrations of the chemical inhibitors which were used in the experiments, caused no significant decrease in cell viability, as analyzed by flow cytometry after incubation with propidium iodide. As shown in Fig. 2, all the inhibitors reduced the antibody-induced internalization of viral glycoproteins in PRV-infected monocytes in a concentration-dependent manner.

Addition of 500 μM colchicine resulted in a significant reduction of internalization of $60.0 \pm 4.5\%$ ($P<0.001$, one way anova) when compared to the non-treated monocytes (relative internalization level of 100%), indicating the importance of microtubules during the antibody-induced internalization of viral glycoproteins. Addition of 30 μM cytochalasin D, 50 μM latrunculin B and 500 μM of amantadine-HCl resulted in a significant reduction of internalization of $79.0 \pm 3.5\%$, $56.0 \pm 1.0\%$ and $59.0 \pm 4.4\%$ respectively ($P<0.001$, one way anova) compared to non-treated cells. These results suggest that actin polymerization, clathrin-coated pit formation and vesicle budding and tubulin polymerization are all involved in the process of antibody-induced internalization of viral glycoproteins.

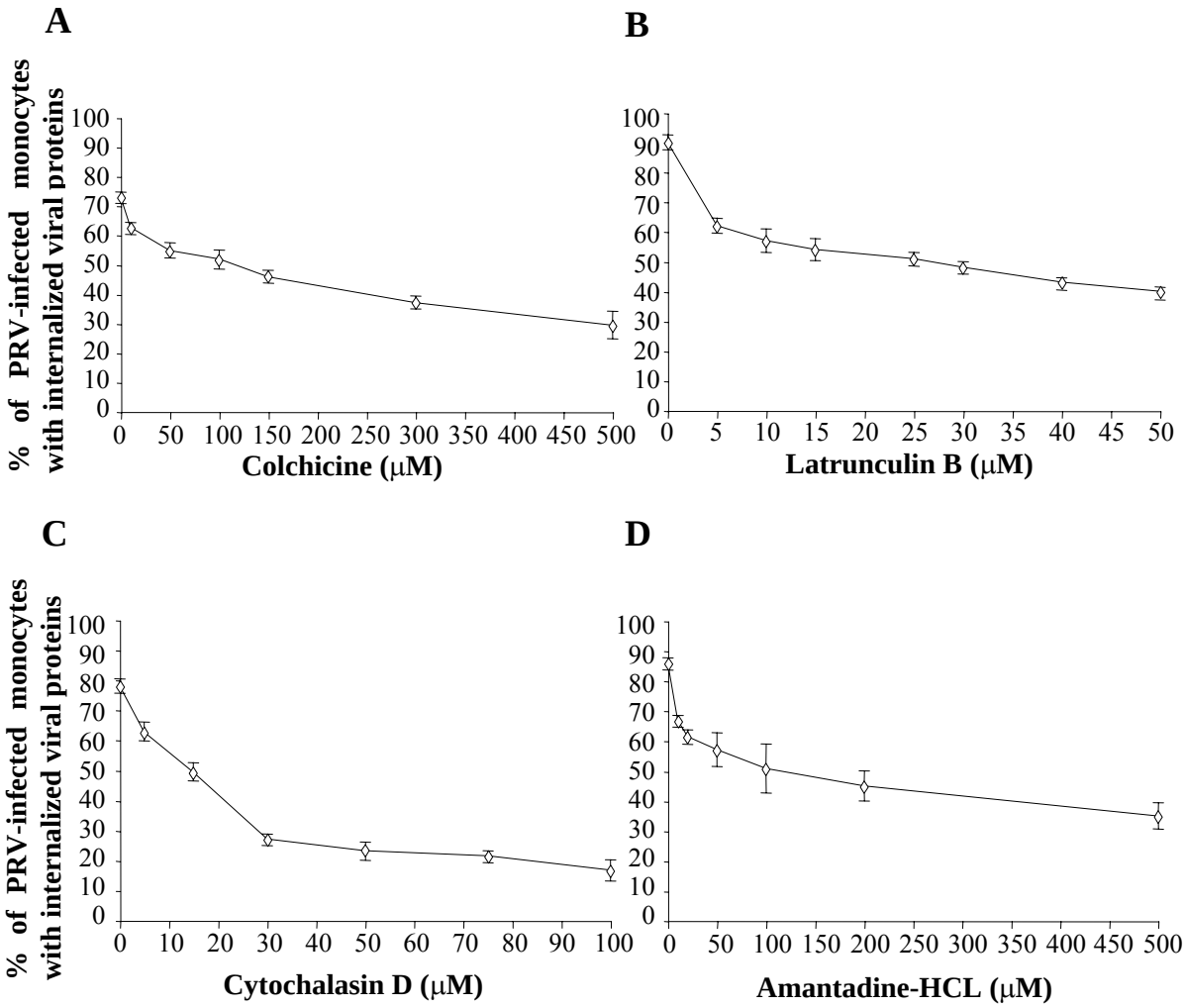

FIG. 2. PRV-infected monocytes (13 h post inoculation) were incubated for 30 min at 37°C with different concentrations of colchicine, an inhibitor of tubulin polymerization (A), cytochalasin D, an inhibitor of G-actin polymerization and clathrin-coated vesicle budding (B), latrunculin B, an inhibitor of actin polymerization (C) and amantadine-HCl, an inhibitor of clathrin-coated pit formation (D). Afterwards, FITC-labeled porcine PRV polyclonal antibodies were added for 1 h at 37°C. The data are means ± standard deviations of triplicate assays.

Visualization of the cellular components microtubules, actin, clathrin and dynein during the different stages of antibody-induced internalization of viral glycoproteins. To further elucidate the role of the different cellular components in the observed process, microtubules, actin, clathrin and dynein were visualized by fluorescence labeling in PRV-infected monocytes at different time points after addition of porcine PRV-specific antibodies.

Microtubules. Monocytes were inoculated with 89V87 for 13 h and incubated with FITC-labeled PRV polyclonal antibodies. After fixation at different time points post antibody addition (p.Ab.a), mouse anti-α-tubulin was added, followed by goat-anti-mouse IgG-Texas Red. The organization of the microtubules in the cell at the different stages during antibody incubation was analyzed by confocal microscopy and scored as an 'intact network' if a clear network with a clear microtubule organizing center (MTOC) was visible and as a 'disorganized network' if only short microtubules and only a vague MTOC were visible. Fig. 3 shows six different configurations of viral glycoproteins and microtubules, observed by confocal microscopy at different time points p.Ab.a. At 0 min p.Ab.a (13 h post inoculation), 96 ± 2.6% of the cells had an intact network with microtubules organized as perinuclear fibers originating from the MTOC. Of the cells in rim and patch, 92 ± 2.6% and 94 ± 2.1% had a disorganized network at 2 and 5 min p.Ab.a respectively. The disorganization of the microtubules was accompanied by a rounding of the cells. Over 99% of the cells with internalized viral proteins showed an intact microtubule network and an intact cell morphology again from 10 up to 60 min p.Ab.a. At these time points (10 up to 60 min p.Ab.a), 94 ± 2.1% and 92 ± 2.4% respectively of the cells that remained in rim and patch had still a disorganized network.

It can be concluded from these results that a depolymerization of the microtubules occurs shortly after antibody binding to the infected cells and that a reorganization of the microtubule network seems to be important for the subsequent internalization of the viral glycoproteins to take place.

Actin. No visible change in the organization of the actin filaments was observed on the different redistribution stages of the viral glycoproteins during PRV-specific antibody incubation. A cortex of actin fibers, lying just beneath the plasma membrane was observed in over 95% of the cells at all time points.

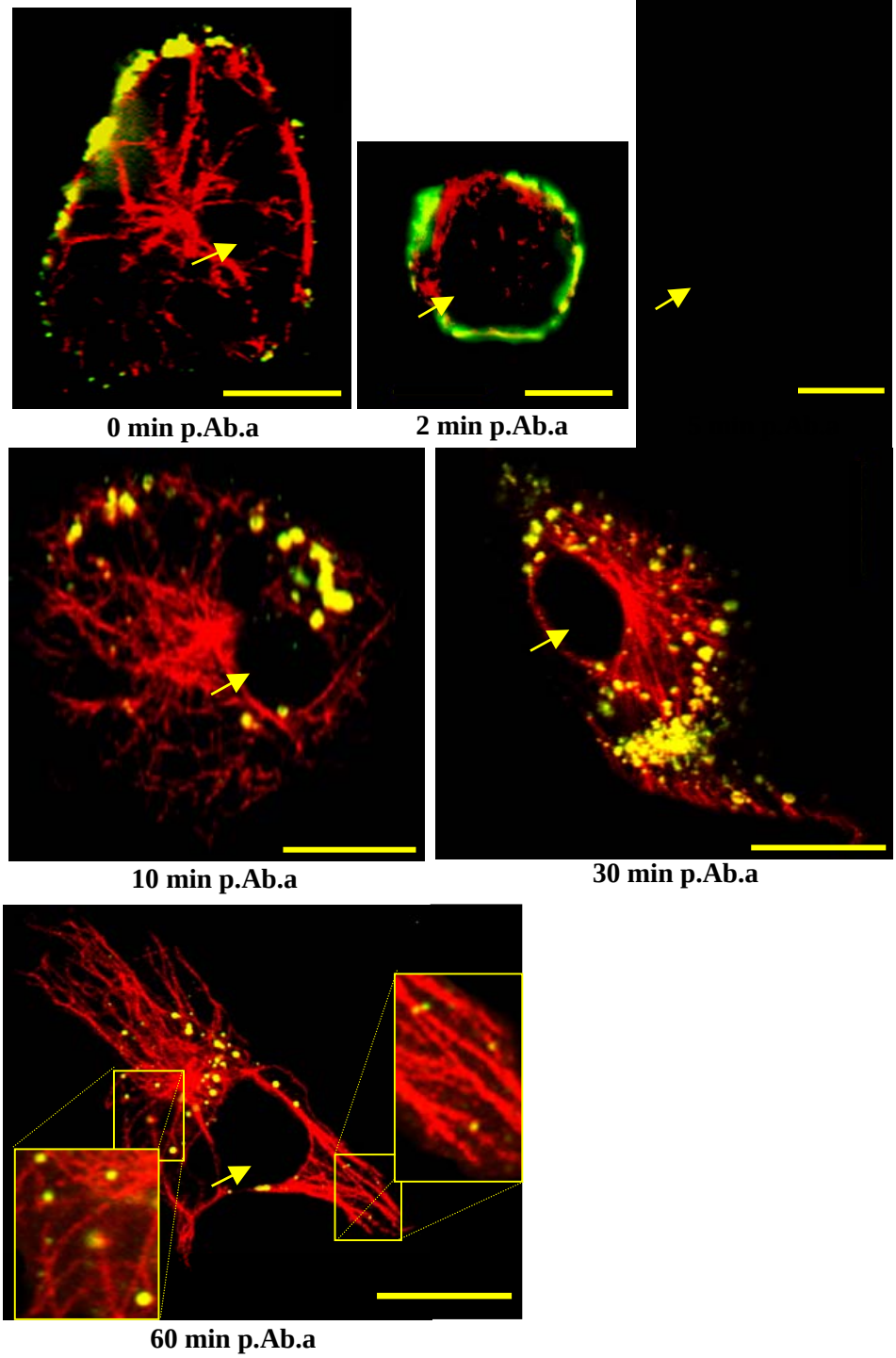

FIG. 3. Double immunofluorescence labeling of viral glycoproteins (FITC) with microtubules (Texas red) at different time points during antibody-induced internalization, observed by confocal microscopy. The images are middle sections of each cell. Arrow indicates the position of the nucleus. Bar, 5 μm.

Clathrin and dynein. With a double immunofluorescence labeling, viral glycoproteins (FITC-labeled) and clathrin or dynein (Texas Red-labeled) were stained to see if and when a co-localization could be observed. First, a positive control with 10 µg of BODIPY FL-LDL (Molecular Probes, Eugene, Oregon) was enclosed, because endocytosis of low-density lipoprotein (LDL) is known to be clathrin- and dynein-dependent (Kibbey *et al.,* 1998; Ichikawa *et al.,* 2000). A co-localization between LDL and clathrin respectively dynein could be observed, indicating that the technique used for the double immunofluorescence labeling experiments was performed in a proper way (data not shown).

Fig. 4A. shows the viral glycoproteins (FITC) with clathrin (Texas Red) at different time points p.Ab.a. An accumulation of clathrin was seen in the regions were the viral glycoproteins were located at 2 and 5 min p.Ab.a. A clear co-localization of vesicles which contain the viral protein-antibody complexes, with clathrin was seen, from 10 till 30 min p.Ab.a. From 30 min p.Ab.a, clathrin started to recycle back to the plasma membrane and the vesicles were further transported towards the nucleus. At 60 min p.Ab.a, a co-localization of the viral glycoproteins with clathrin was no longer observed.

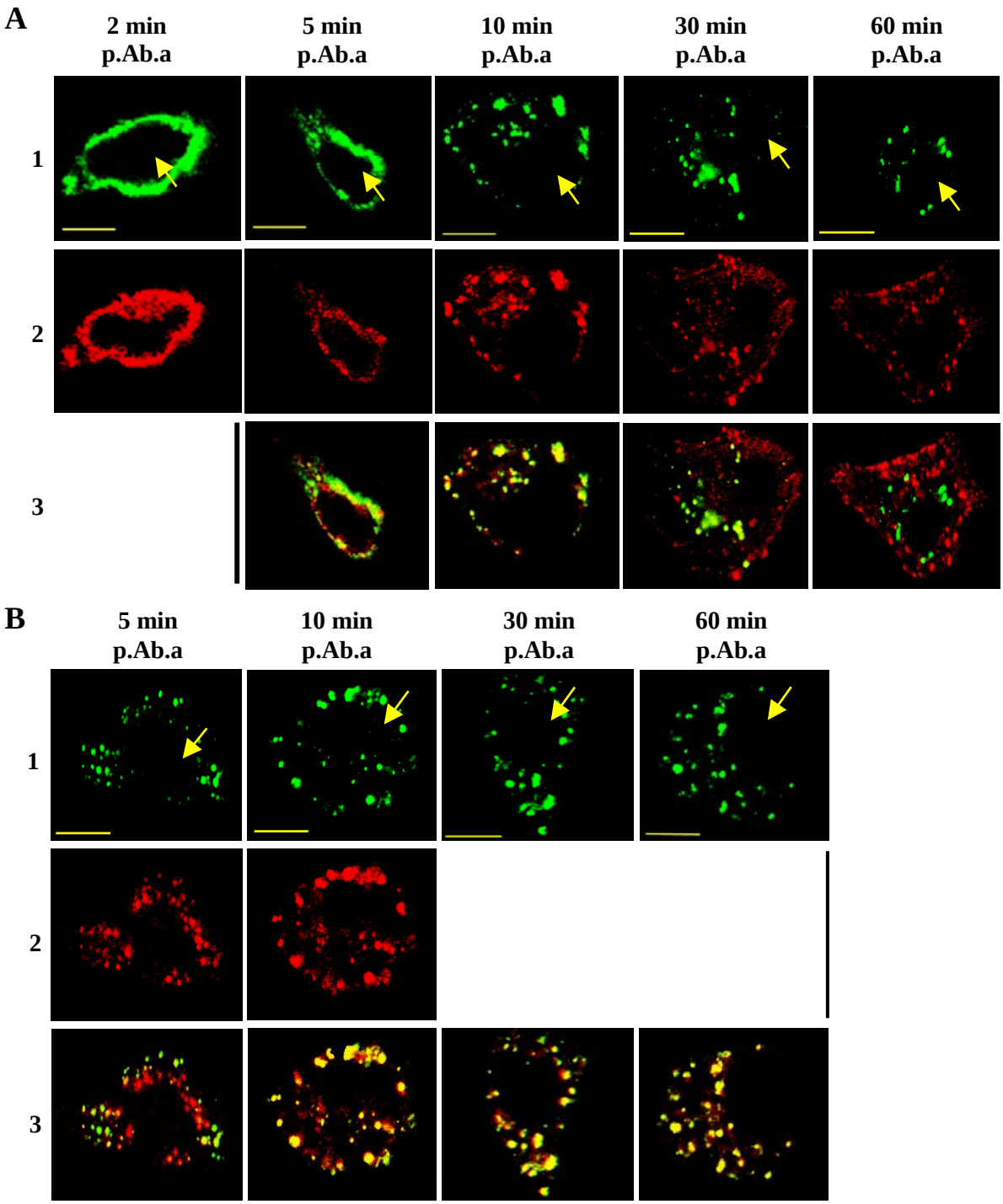

FIG. 4. Double immunofluorescence labeling of viral glycoproteins (FITC) with clathrin (Texas red) (A) and dynein (Texas red) (B) at different time points during antibody-induced internalization, observed by confocal microscopy. Lane 1 shows viral proteins, lane 2 shows clathrin (A) and dynein (B) and lane 3 shows images, obtained by merging (1) and (2). The images are middle sections of each cell. Arrow indicates the position of the nucleus. Bar, 5 μm.

Discussion

PRV-infected porcine blood monocytes have been shown to be capable of transporting the virus to different internal organs in pigs with specific antibodies in the blood circulation (Wittmann *et al*., 1980; Nauwynck & Pensaert, 1992; Nauwynck & Pensaert, 1995a). Since viral proteins become expressed on the plasma membrane of PRV-infected monocytes, one would expect that antibody-dependent cell lysis will eliminate these cells. However, some cells avoid such destruction. One possible explanation for the inefficient antibody-dependent lysis of PRV-infected monocytes has been described recently: the fast and efficient antibody-induced internalization of viral cell surface proteins (Favoreel *et al.*, 1999).

The data presented here show that this antibody-induced internalization process shows strong similarities to the bivalent ligand-induced endocytosis of cellular receptors (Goldstein *et al*., 1985). In order to generate the antibody-viral antigen internalization process, the PRV-infected monocyte mobilizes different cellular components such as clathrin, actin, dynein, and microtubules as shown by the use of different chemical inhibitors and by co-localization studies between the different cellular components and the viral proteins.

The first step after the addition of PRV-specific antibodies to PRV-infected monocytes is the aggregation of the viral plasma membrane glycoproteins. This aggregation (patching) is followed by an invagination of the plasma membrane and the formation of vesicles, containing the viral protein-antibody complexes. The importance of clathrin during these early steps was shown by inhibition of clathrin-coated-pit invagination at the plasma membrane using amantadine-HCl and inhibition of clathrin-coated vesicle budding using cytochalasin D which resulted in a strong decrease in internalization, as well as by immunofluorescence double staining. A clear co-localization of vesicles which contain the viral glycoprotein-antibody complexes and clathrin was seen from 5 till 30 min p.Ab.a. From that time point on, clathrin recycled back to the plasma membrane and the vesicles were further transported towards the nucleus. A very similar role for clathrin has been shown during the spontaneous endocytosis of certain herpes viral glycoproteins, such as varicella-zoster virus gE (Olson & Grose, 1997) and human cytomegalovirus virus gB (Tugizov *et al.*, 1999). Spontaneous endocytosis of PRV gB and gE has also been described and is

likely to be clathrin-dependent as well (Tirabassi & Enquist, 1998). Despite these similarities, it is, in the current context, important to clearly discriminate spontaneous gB and gE endocytosis and the antibody-induced internalization of the major viral cell surface proteins. PRV gB and gE endocytosis are spontaneous processes (antibody-independent), which affect gB and gE alone and which occur during early stages of infection (< 6 h post infection) (Tirabassi & Enquist, 1998). Antibody-induced internalization of PRV proteins, on the other hand, is antibody-dependent, results in internalization of all major viral cell surface proteins, and can be induced from 7 up to 17 h post infection (Favoreel *et al.,* 1999).

Actin filaments have also been shown to be of major importance during the first stages of the receptor-mediated endocytosis process, but a clear role for actin in endocytic processes has not been established (reviewed by Qualmann *et al.,* 2000). Two major hypotheses have been described. The first hypothesis proposes an active role of actin during the initial stages of the endocytosis process, by which actin modulates vesicle budding and mediates vesicle transport or fusion (Lamaze *et al.,* 1997; Merrifield *et al.,* 1999). The second hypothesis suggests a more passive role for actin filaments, by which actin provides the integrity of the cell, necessary for the endocytosis process to take place (Gaidarov *et al*., 1999). Since a significant reduction in endocytosis was observed with both the inhibitors cytochalasin D and latrunculin B, but no change in distribution of actin filaments was observed during the different stages of the internalization process by fluorescence labeling, our results tend to favour the second hypothesis.

During bivalent ligand-induced endocytosis of cellular receptors, the clathrin-coated pits are pinched off into the cell as clathrin-coated vesicles, followed by shedding of the clathrin coat and fusion of the endocytic vesicles with early endosomes. Further transport of these vesicles towards the nucleus is mediated by dynein, a microtubule motor protein that moves along microtubules (Goodson *et al*., 1997; Hamm-Alvarez, 1998). In the present study, involvement of dynein in the transport of the vesicles containing the viral protein-antibody complexes was demonstrated by a clear co-localization of the internalized viral proteins with dynein from 5 till 60 min p.Ab.a.

In order to have an indication whether the microtubules function as ‘highways’ during this transport of the vesicles towards the cell nucleus, experiments with a chemical inhibitor of microtubule polymerization (colchicine) and double immunofluorescence labeling of microtubules with the viral proteins were performed. Addition of

colchicine resulted in a significant decrease in internalization, which suggests the involvement of microtubules during the process of antibody-induced internalization of viral glycoproteins. A remarkable observation was made when microtubules and viral glycoproteins were analyzed by double immunofluorescence staining. At 0 min p.Ab.a, the cells had an intact network and normal cell morphology, indicating that an infection with PRV had no influence on the organization of the microtubule network. This observation is consistent with earlier experiments performed at our laboratory, which revealed that the microtubule network remains intact from 0 until 16 hours after infection with 89V87 (data not shown). During the stages of rim and patch (2 and 5 min p.Ab.a respectively), the microtubule network became disorganized which was accompanied by a loss of cell shape. It has already been described that a disorganization of the microtubule network affects the cell shape of certain cell types, including macrophages (Rodinov *et al*., 1993; Rosania & Swanson, 1996). During the subsequent stages of internalization (10, 30 and 60 min p.Ab.a), the microtubule network appeared to be intact again, accompanied by a convalescent normal cell shape and transport of the vesicles towards the nucleus. It has been shown that certain membrane proteins are prevented from patching and subsequent endocytosis because they are linked to microtubules. These integral membrane proteins are immobilized at specialized microdomains on the cell surface, which are linked to the microtubules via the spectrin-ankyrin network, a member of the membrane-associated cell cortex (Drenckhahn *et al*., 1993). Furthermore, it has been suggested that the size of bivalent antigen-induced aggregates (patches) of cellular plasma membrane proteins needs to exceed a 'minimal patch size' before subsequent endocytosis can occur (Bourguignon & Bourguignon, 1984). Indications for the existence of a similar minimal patch size for antibody-induced internalization of PRV cell surface proteins to occur have been described (Favoreel *et al.,* 1999). It could be hypothesized that some or all of the viral proteins inserted in the plasma membrane are (in)directly linked with and immobilized by components of the cellular cytoskeleton, such as microtubules. Such an association has already been described for the Us9 glycoprotein of the human cytomegalovirus, a betaherpesvirus (Maidji *et al.,* 1998). If future research shows that a similar cytoskeleton-association exists for PRV glycoproteins as well, disorganization of the microtubule network would be necessary for proper patching and subsequent internalization to occur.

The underlying physiological mechanism explaining the microtubule disorganization remains to be studied. One possible explanation could be that antibody-induced aggregation of viral cell surface proteins induces a Ca2+ influx in the monocytes. Such a Ca2+ influx has already been described upon aggregation of cellular receptors by bivalent ligands and antibodies (Bourguignon & Bourguignon, 1984; Dyer & Benjamins, 1990). Since high concentrations in Ca2+ are known to destabilize the microtubule network, such a Ca2+ influx could cause the observed microtubule disorganization (Soto *et al.,* 1996).

It is generally accepted that microtubules are being used by cells for efficient transport of proteins and lipids to various sites within the cell (Cole & Lippincott-Schwartz, 1995). Therefore it is reasonable to suggest that the microtubule network needs to be intact again before transport of the internalized viral glycoproteins towards the center of the cell can take place. This is consistent with the results of the present study where it was shown that the microtubules regained their structure at approximately 10 min p.Ab.a, during the later stages of the internalization process. The low percentage of cells in rim and patch that remained in these configurations during these later stages p.Ab.a still had a disorganized microtubule network, supporting the idea that an intact network is necessary for later stages in the internalization process to occur.

In conclusion, the current study provides strong indications that actin, microtubules, clathrin and dynein, important components during physiological endocytosis, are being mobilized by PRV-infected monocytes to generate the antibody-induced internalization of viral glycoproteins, a potential immune evasion mechanism of PRV. Furthermore, the present findings establish an interesting *in vitro* model for (biochemical) investigations on the possible interactions between certain viral glycoproteins, expressed on the plasma membrane of a PRV-infected cell, and different components of the cellular cytoskeleton. The model can also be used to study viral and cellular endocytosis processes in general, since the antibody-induced internalization of viral glycoproteins in PRV-infected monocytes is easily reproducible and is a fast, very efficient and coordinated process.

Acknowledgments

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4.

PSEUDORABIES VIRUS-SPECIFIC ANTIBODIES SUPPRESS INTRACELLULAR VIRAL PROTEIN EXPRESSION IN PSEUDORABIES VIRUS-INFECTED MONOCYTES

Journal of General Virology, conditionally accepted

Summary

Although rare, pseudorabies virus (PRV) may cause abortion in the presence of a vaccination-induced immunity and blood monocytes have been shown to be essential to transport the virus throughout the body of these immune animals. It has been demonstrated that addition of PRV-specific polyclonal antibodies to PRV-infected monocytes induces internalization of plasma membrane-anchored viral proteins (immune-masked monocytes), a process that interferes with antibody-dependent cell lysis *in vitro*. In the present study, we examined the long-term fate of these productively infected immune-masked monocytes with regard to viability of the cells and expression of viral antigens. In a first assay, the viability of PRV-infected monocytes in the presence or absence of PRV-specific antibodies was evaluated until 194 h post inoculation (p.i.). It was shown that in the continuous presence of PRV-specific antibodies, approximately 30% of the PRV-infected monocytes were still viable at 194 h p.i., compared to 0% in the absence of PRV-specific antibodies. In a second assay, the expression of viral glycoproteins was investigated in the immune-masked monocytes, in the continuous presence of PRV-specific antibodies until 194 h p.i. This assay showed that approximately 75% of the surviving immune-masked PRV-infected monocytes did no longer show visually detectable expression of viral proteins from 120 h p.i. onward. Addition of the reactivating agent dexamethasone was able to induce a re-expression of viral proteins. In conclusion, it can be stated that internalization of viral cell surface proteins in PRV-infected monocytes may not only delay destruction of the cells by the immunity, but may also lead to a quiescent infection and an expanded life span of the cells, giving a possible explanation for how these cells may act as carriers of PRV in the blood of vaccinated animals.

Introduction

The alphaherpesvirus pseudorabies virus (PRV) causes Aujeszky's disease in its natural host, the pig. The disease is characterized by nervous signs in newborn pigs, respiratory disorders in fattening pigs and reproductive failure in sows (Wittmann *et al.*, 1980). Abortion can be an important consequence of PRV infection in susceptible pregnant sows (Pensaert & Kluge, 1989). Even in the presence of a vaccination-induced immunity, PRV inoculation may result in infection of the respiratory tract, involving mononuclear cells in draining lymph nodes. These cells can enter the bloodstream, resulting in a restricted viraemia (Wittmann *et al*., 1980), that generally does not cause problems. However, rarely, abortion in immune animals can occur as a result of transplacental spread and intrafetal replication. Infected porcine blood monocytes are essential to transport the virus in vaccination-immune pigs to enable the virus to reach the pregnant uterus (Nauwynck & Pensaert, 1992; Nauwynck & Pensaert, 1995a). Different components of the immunity, including the antibody-dependent immune effectors, should be able to lyse PRV-infected monocytes in the blood stream of vaccinated animals. Indeed, PRV-infected monocytes express viral proteins in their plasma membrane (Favoreel *et al.*, 1999) and binding of antibodies to these antigens should induce antibody-dependent lysis of these cells (Sisson & Oldstone, 1980). One of the mechanisms that may aid PRV in masking infected blood monocytes from antibody-dependent cell lysis consists of the antibody-induced internalization of antigen-antibody complexes (Favoreel *et al.,* 1999). During this process, binding of PRV-specific antibodies to viral proteins on the cell surface of PRV-infected monocytes results in internalization of the antigen-antibody complexes (immune-masked monocytes). This internalization process is fast and efficient, mediated by viral proteins gB and gD, and reduces the sensitivity of PRV-infected monocytes towards antibody-dependent complement-mediated cell lysis *in vitro* (Van de Walle *et al.*, 2001; 2003a). Such immune-masked monocytes have already been shown to be capable of transmitting virus to vascular endothelial cells through adhesion and fusion processes in the presence of neutralizing antibodies *in vitro*, as a possible first step for PRV to reach internal organs (Van de Walle *et al.*, 2003b).

To date, antibody-induced internalization of viral cell surface proteins has only been studied *in vitro* over relatively short periods post antibody addition (≤ 2 h) and post

inoculation (≤ 17 h). However, the circulation of PRV-infected monocytes *in vivo* occurs in the continuous presence of PRV-specific antibodies. Therefore, the aim of the current study was to investigate the effect of the continuous presence of porcine PRV-specific antibodies on PRV-infected monocytes over an extended period of time, examining both the viability of the infected cells as well as the expression level of viral proteins in the infected cells.

Materials and Methods

Virus strains. PRV strain 89V87 was used in all experiments (Nauwynck & Pensaert, 1992).

Isolation of porcine blood monocytes. Pigs from a PRV-negative farm were used as blood donors. Blood was collected from the *vena jugularis* on heparin (15 U/ml) (Leo, Zaventem, Belgium). Blood mononuclear cells were separated on Ficoll Paque (Pharmacia Biotech AB, Uppsala, Sweden) following the manufacturer's instructions. Mononuclear cells were then resuspended in medium A, based on RPMI-1640 (Gibco BRL, Life Technologies Inc., Gaithersburg, MD) supplemented with 10% fetal calf serum (FCS), 0.3 mg/ml glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.1 mg/ml kanamycin, 1 mM sodium pyruvate, 1% non-essential amino acids 100x (Gibco BRL) and 10 U/ml heparin. Afterwards, cells were seeded on 4-well multidishes (Nunc A/S, Roskilde, Denmark) at a concentration of $2.5x10^6$ cells/ml and cultivated at 37°C with 5% CO_2. After 48h, non-adhering lymphocytes were removed by washing the 4-well multidish three times with RPMI-1640.

Inoculation of blood monocytes. The adherent cells, consisting of ≥70% of monocytes (as assessed by flow cytometric analysis using the monoclonal antibody 74.22.15 (Pescovitz *et al.*, 1984)), were inoculated 3 times with a time interval of 24 h with the PRV strain 89V87 at a m.o.i. of 10 in 0.5 ml medium A without heparin. Mock-infected monocytes were used as a control.

Incubation of PRV-infected monocytes with porcine antibodies. Monocytes, were always at 1 h post inoculation (p.i.) or mock-infected, washed three times with RPMI-1640 and incubated with porcine PRV-specific polyclonal antibodies (0.4 mg of IgG/ml). These protein A-purified IgG antibodies were, as described earlier (Nauwynck & Pensaert, 1995b), derived from a PRV (89V87)-inoculated pig originating from a PRV-negative farm and had a titer of 512, as determined with a serum neutralization (SN) test (Andries *et al.*, 1978). As a control, protein A-purified PRV-negative porcine serum was used, at the same concentration (0.4 mg of IgG/ml). This serum was derived from a PRV-negative pig and had a titer < 2 with the SN test.

At different time points (0, 24, 48, 72, 96, 120 and 194 h) p.i., monocytes were incubated for 30 min on ice with 100 μg/ml of ethidium mono-azide bromide (EMA) (Molecular Probes, Eugene, Oregon, USA) which specifically stains dead cells. Cells were washed twice with RPMI-1640 and cell smears were made by centrifuging for 4 min at 700 rpm, followed by fixation for 20 min at –20°C with acetone 100%.

Direct immunofluorescence staining of cell smears. Cell smears were washed twice with phosphate buffered saline (PBS) and viral antigens were visualized by incubating cell smears for 1 h at 37°C with FITC-labeled porcine polyclonal IgG antibodies (Favoreel *et al.*, 1999), dilution 1:100 in PBS. After washing twice in PBS, nuclei were stained by incubating cell smears at room temperature for 10 min with Hoechst 33342 (Molecular Probes, Eugene, Oregon, USA) which stains nuclei, dilution 1:100 in PBS. Finally, cell smears were washed thoroughly with PBS, mounted with a glycerin-phosphate buffered saline solution (PBS) (0.9:0.1, vol/vol) with 2.5% 1,4-diazabicyclo (2.2.2) octane (DABCO; Janssen Chimica, Beerse, Belgium), excited with an Osram HBO 50-W bulb using an I3, a TR and a D filter, and observed with a Leica DM RBE microscope (Leica GmbH, Wetzlar, Germany). Quantitative results were obtained by examining the EMA (dead cells), Hoechst (all cells) and FITC intensity (viral antigen-positive cells). Images were obtained by using a Sony color video camera (model DXC-9100P), linked to a Macintosh computer. Statistical analysis were performed with SPSS (SPSS Inc., Chicago, Illinois,USA).

Treatment of PRV-infected monocytes with dexamethasone. At 144 h p.i., PRV-infected monocytes incubated with PRV-specific polyclonal antibodies were washed three times with PRMI-1640 and subsequently incubated with 3 μM dexamethasone 98% (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), supplemented with 0.4 mg/ml PRV-specific polyclonal antibodies. At two and four days after the addition of dexamethasone, (at 192 and 240 h p.i. respectively), cells were incubated with EMA, cell smears were made and direct immunofluorescence stainings were performed as described above.

Results

Efficiency of PRV inoculation of monocytes in the continuous presence or absence of PRV-specific antibodies. Figure 1. shows that the repeated PRV inoculation as described in the Materials and methods section resulted in the expression of viral antigens in all monocytes at 96 h p.i., both in the presence and in the absence of PRV-specific antibodies, as determined by immunofluorescence staining with FITC-labeled PRV-specific antibodies. Hence, all monocytes became productively infected irrespective of the presence of PRV-specific antibodies in the medium.

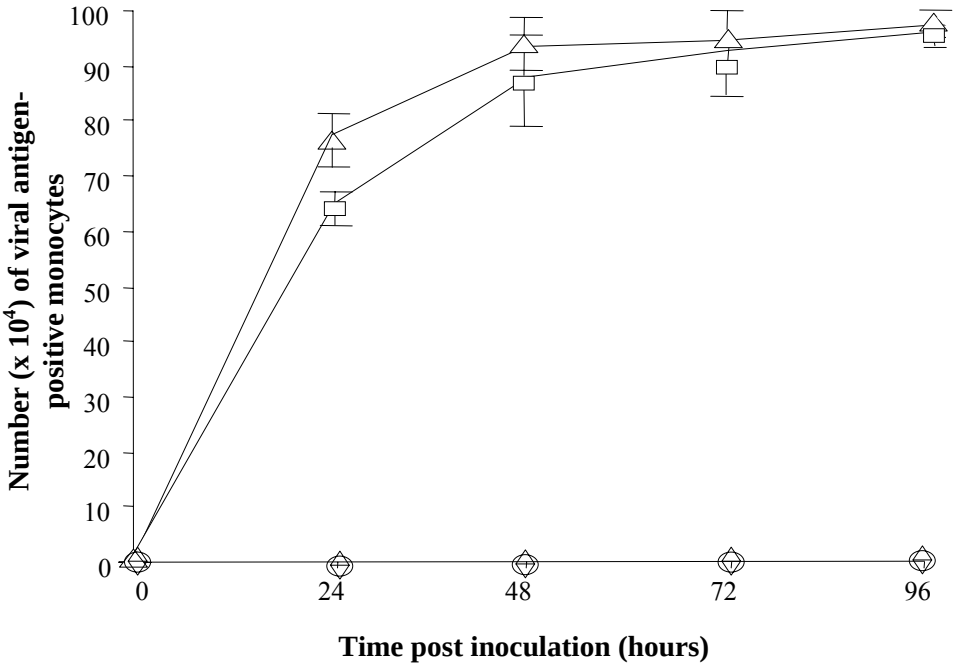

FIG. 1. Efficiency of PRV inoculation. Monocytes (n= 10^4) were inoculated three times with PRV at a time interval of 24 h, and, each time, at 1 h p.i., supplemented with either 0.4 mg/ml PRV-specific polyclonal IgG antibodies (□) or 0.4 mg/ml PRV-negative IgG antibodies (Δ). Mock-infected monocytes, incubated with either PRV-specific (◊) or PRV-negative (o) IgG antibodies were used as controls. At different time points p.i., expression of viral antigens was determined by fixation of the cells for 20 min at –20°C in acetone 100%, followed by incubation for 1 h at 37°C with FITC-labeled PRV-specific porcine polyclonal antibodies. Data represent means ± standard deviations of triplicate assays.

Long term survival of monocytes in the continuous presence or absence of PRV-specific antibodies. To evaluate whether and how long PRV-infected monocytes survive in the continuous presence of antibodies, monocytes were inoculated 3 times with the PRV strain 89V87 and always at 1 h p.i. resuspended in

medium supplemented with 0.4 mg/ml of porcine PRV-specific polyclonal IgG antibodies or, as a control, 0.4 mg/ml PRV-negative porcine IgG. Mock-infected monocytes, incubated with either PRV-specific or PRV-negative IgG antibodies were used as controls. Fig. 2 shows that the viability of PRV-infected monocytes, both in the presence or absence of PRV-specific antibodies, decreased dramatically until 72 h p.i. The percentage of viable cells at different time points post inoculation in the presence of PRV-negative IgG antibodies was comparable with the percentage of viable cells at the corresponding time points in the absence of antibodies (data not shown). However, from 120 h p.i. onwards, all PRV-infected monocytes incubated with PRV-negative IgG antibodies were lysed, whereas approximately 30% of the PRV-infected monocytes incubated with PRV-specific polyclonal IgG antibodies remained viable (box). These results indicate that in the continuous presence of PRV-specific antibodies, some PRV-infected monocytes remain viable for unusual long periods of time.

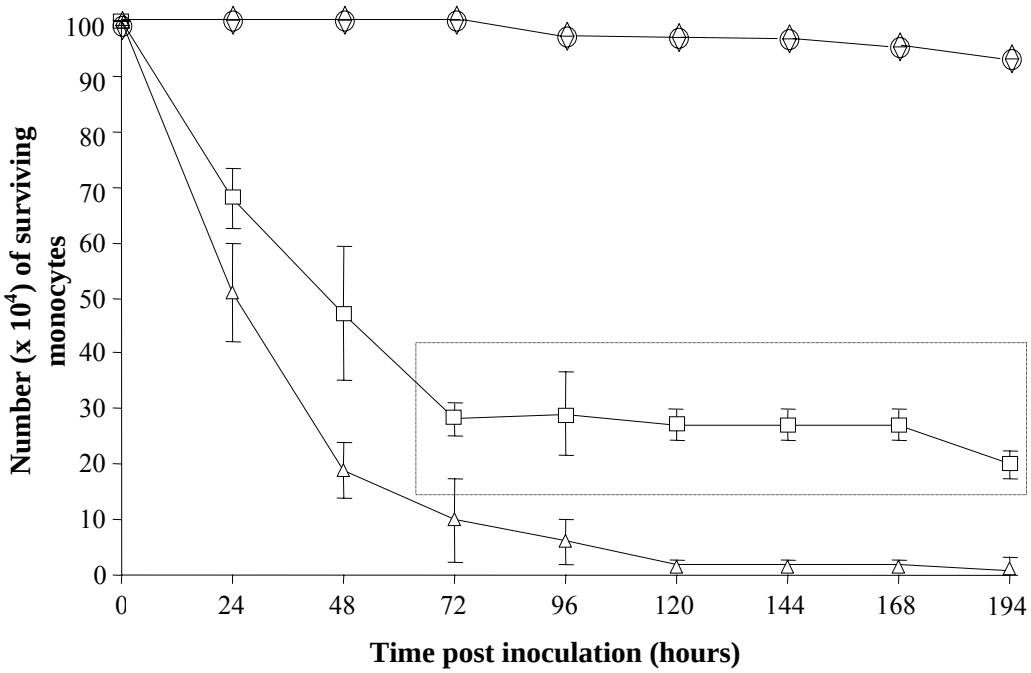

FIG. 2. Long term survival of PRV-infected monocytes. Monocytes (n= 10^4), at 1 h post inoculation with PRV, were incubated with 0.4 mg/ml of either PRV-specific polyclonal IgG antibodies (□) or PRV-negative IgG antibodies (Δ). Mock-infected monocytes, incubated with either PRV-specific (◊) or PRV-negative (o) IgG antibodies were used as controls. At different time points p.i., viability was determined by incubating the cells for 30 min on ice with ethidium mono-azide bromide (EMA) to stain dead cells and, after fixation in acetone 100%, all cells were stained by incubating for 10 min at room temperature with Hoechst. Data represent means ± standard deviations of triplicate assays.

Expression of viral proteins in the long term surviving PRV-infected monocytes. In this part of the study, the expression of viral antigens was examined in

the subpopulation of PRV-infected monocytes that survived for unusual long times in the presence of PRV-specific antibodies (Fig. 2, box). Fig. 3A shows that the majority of these surviving PRV-infected cells gradually stopped producing viral antigens. All of the surviving monocytes were positive for viral antigens at 72h p.i., whereas, by 194 h p.i., only 25% of the surviving cells showed visually detectable viral antigen expression.

Fig. 3.B shows monocytes, at 194h p.i. with PRV, in the continuous presence of PRV-specific polyclonal IgG antibodies. Of the three PRV-infected monocytes (all three nuclei are stained with Hoechst), two cells are no longer viable, as determined by EMA (indicated by arrows), and show viral glycoprotein expression (visualized by incubation with FITC-labeled PRV-specific antibodies). The third, surviving PRV-infected cell, shows no or very little visually detectable viral protein expression. The results from these experiments demonstrate that in the continuous presence of PRV-specific antibodies, the majority of surviving PRV-infected monocytes stop expressing visually detectable viral antigens.

Effect of dexamethasone addition. The observation that, in the continuous presence of PRV-specific antibodies, approximately 75% of the surviving PRV-infected monocytes do no longer express visually detectable viral antigens from 120 h p.i. onwards could imply that antibody binding to the viral antigens on the cell surface of PRV-infected monocytes may abort the infection and direct the cells in a quiescent infected state. To investigate whether this stage of quiescence possibly resembles latency, dexamethasone, a glucocorticoid known to induce herpesvirus reactivation from latency (Schoembaum *et al.*, 1990; Donofrio & van Santen, 2001), was added to the cells. Therefore, monocytes at 144h p.i. with PRV and in the continuous presence of 0.4 mg/ml PRV-specific polyclonal IgG antibodies, were washed and 3 μM dexamethasone was added, supplemented with 0.4 mg/ml PRV-specific polyclonal IgG antibodies.

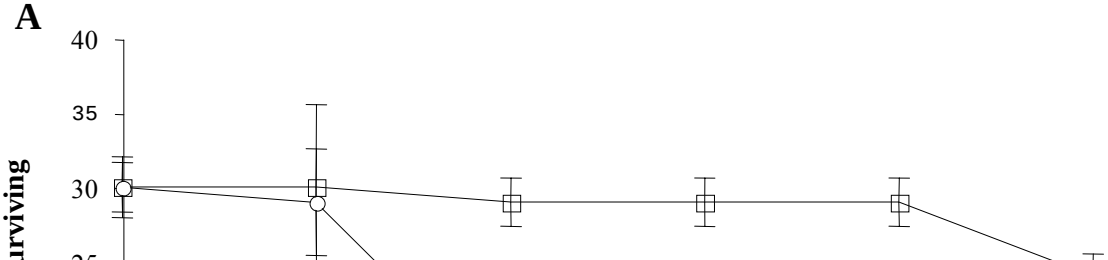

FIG. 3. (A). Expression of viral glycoproteins in the long term surviving PRV-infected monocytes. Monocytes (n= 10^4), at 1 h post inoculation with PRV, were incubated with 0.4 mg/ml of PRV-specific polyclonal IgG antibodies. From 72 h p.i. onwards, expression of viral proteins in surviving PRV-infected monocytes was determined at different time points as described in Materials and Methods. The square represents the total number (x 10^4) of surviving monocytes. The number of surviving monocytes with visually detectable viral antigen expression (o) gradually decreases whereas the number of surviving monocytes without visually detectable viral antigen expression (Δ) increases over time. Data represent means ± standard deviations of triplicate assays.

(B). Immunofluorescence staining of monocytes at 194 h p.i. with PRV, in the continuous presence of PRV-specific antibodies. Arrows indicate dead cells with abundant viral protein expression, arrowheads indicates living cells with low viral protein expression. Bar 3 μm.

The concentration of dexamethasone used had no effect on cell viability, as assessed by flow cytometric analysis using 10 μg propidium iodide (Molecular Probes, Eugene, Oregon, USA) which specifically stains dead cells (data not shown).

addition of dexamethasone at t=144 h p.i.	Number (x 10^4) of surviving monocytes with viral antigen expression at ... post inoculation		
	t=144	t=192	t=240
no	7.0 ± 2.3	6.0 ± 1.2	5.0 ± 1.7
yes	7.0 ± 2.3	**13.0 ± 1.5***	**14.0 ± 0.6***

Table 1. Effect of dexamethasone on the number of surviving PRV-infected monocytes with viral antigen expression. Monocytes, at 144 h post inoculation with PRV and in the continuous presence of 0.4 mg/ml of PRV-specific polyclonal IgG antibodies, were washed and incubated with 3 μM dexamethasone 98%, supplemented with 0.4 mg/ml PRV-specific polyclonal antibodies. At 192 h and 240 h p.i., expression of viral glycoproteins in surviving PRV-infected monocytes was determined. Data represent means ± standard deviations of triplicate assays. Asterisks indicate significant differences ($p < 0.01$, one way anova).

At two (192 h p.i.) and four (240 h p.i.) days after the dexamethasone addition, PRV-infected monocytes were analyzed for the number of surviving cells that were viral antigen-positive (the total number of surviving cells remained relatively constant, data not shown). The results of these experiments are presented in Table 1. When no dexamethasone was added to monocytes in the presence of PRV-specific antibodies, the number of surviving cells with viral antigen expression did not alter significantly (control). However, after the addition of dexamethasone, supplemented with PRV-specific antibodies, the number of surviving viral antigen-positive monocytes started to significantly increase from 7.0 ± 2.3 x 10^4 to 13.0 ± 1.5 x 10^4 at two days and 14.0 ± 0.6 x 10^4 at four days after the dexamethasone addition ($p < 0.01$, one way anova).

Discussion

The main purpose of this study was to investigate *in vitro* the fate of PRV-infected monocytes with antibody-induced internalization of the plasma membrane-anchored viral proteins (immune-masked monocytes) for extended periods of time in the continuous presence of PRV-specific antibodies. Therefore, both the viability of these PRV-infected immune-masked monocytes and the expression of viral proteins was examined until 194 h p.i. in the continuous presence of PRV-specific or PRV-negative antibodies.

It was shown that, although a productive PRV infection normally leads to cell lysis starting at 48-72 h (Wang *et al.*, 1988; present results), in the continuous presence of PRV-specific antibodies approximately 30% of the productively infected monocytes remained viable for an unusual long period of time (up to 194 h after inoculation with PRV, end of the experiment). Although carried out in an *in vitro* model, these results may indicate that immune-masked PRV-infected monocytes *in vivo* (continuously surrounded by PRV-specific antibodies in the blood of vaccinated animals) may have a prolonged life time, giving these cells more chance to spread throughout the body of vaccination-immune pigs.

Analyzing the expression of viral proteins in the immune-masked PRV-infected monocytes (cultivated in the continuous presence of PRV-specific antibodies) that survived a PRV-infection for unusual long periods of time, led to a somewhat surprising observation. The number of surviving monocytes with visually detectable antigen expression, being $3.0x10^5$ at 96 h p.i., significantly decreased from that time point onward to approximately $0.5x10^5$ at 194 h p.i.. This result implies that the continuous presence of PRV-specific antibodies leads to a quiescent viral infection in a subpopulation of PRV-infected monocytes. Suppression of intracellular virus replication upon binding of specific antibodies to infected cells has already been reported for herpes simplex virus type 1 (HSV-1) and Sindbis virus. Binding of HSV-specific antibodies, especially anti-gB and anti-gE antibodies, to HSV-1-infected neuronal cells results in a quiescent intracellular expression of viral proteins (Oakes & Lausch, 1984; Sanna *et al.*, 1999). Moreover, for Sindbis virus it was demonstrated that suppression of intracellular virus expression upon antibody binding resulted in a prolonged cell survival (Chanas *et al.*, 1982). The fact that mainly gB- and gE-

specific antibodies are capable of suppressing intracellular HSV-1 replication is remarkable since we showed earlier that PRV gB and gE can activate intracellular signal transduction pathways upon antibody binding (Favoreel *et al.*, 1997; 2002). Further research will be necessary to clarify if antibody-induced activation of intracellular signal transduction pathways may lead to a quiescent PRV infection.

Moreover, our observation that a significant fraction of the PRV-infected cells without visually detectable viral expression can be stimulated to resume viral antigen expression upon addition of the reactivating agent dexamethasone, suggests the intriguing possibility that the antibody-induced quiescent state of PRV-infection may resemble or lead to a latent PRV infection. Latency is defined as a status in which viral DNA persists, but infectious virus is not produced, and is a well-known characteristic of herpesviruses, including PRV (Beran *et al.*, 1980). For PRV it is known that the virus mainly establishes latency in neurons of the trigeminal ganglion (Wittmann *et al.*, 1983; Cheung, 1995). However, latency in non neuronal sites, including tonsils and blood mononuclear cells, has also been suggested for PRV (Wittmann & Rziha, 1989). Our observation that the antibody-induced quiescent PRV infection in monocytes may possibly resemble latency may aid in clarifying this issue. However, it is crucial to point out that our dexamethasone data may be indicative, but not more than that. Dexamethasone is a member of the glucocorticoid family which have numerous effects on the cell. It is generally accepted that most, if not all, of the effects of glucocorticoids are mediated via the glucocorticoid receptor, which will undergo a conformational change upon ligand binding which in turn ultimately leads to activation of the transcription of a variety of responsive genes (Newton, 2000). Hence, although some of the responses induced by dexamethasone may induce herpesvirus reactivation, several other responses may possibly give alternative explanations for our observations. Further experimentation, consisting of in situ hybridisation using RNA probes detecting on the one hand gB-mRNA (as a tool to detect productively infected cells) and on the other hand LAT-RNA (to detect latently-infected cells) are a first necessity to investigate whether the PRV quiescent infection in monocytes resembles a latent infection.

Taken all the results from this study together, it can be concluded that internalization of viral cell surface proteins in PRV-infected monocytes may not only delay destruction of the cells by the immunity, but may also lead to a quiescent

infection and an expanded life span of the cells, giving these cells the opportunity to reach internal organs via the blood of vaccinated animals.

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5.

TRANSMISSION OF PSEUDORABIES VIRUS FROM IMMUNE-MASKED BLOOD MONOCYTES TO ENDOTHELIAL CELLS

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Summary

Pseudorabies virus (PRV) may cause abortion, even in the presence of a vaccination-induced immunity. Blood monocytes are essential to transport the virus in these immune animals, including transport to the pregnant uterus. Infected monocytes express viral proteins on their cell surface. Specific antibodies recognize these proteins and should activate antibody-dependent cell lysis. Previous work showed that addition of PRV-specific polyclonal antibodies to PRV-infected monocytes induced internalization of viral cell surface proteins, protecting the cells from efficient antibody-dependent cell lysis *in vitro* (immune-masked monocytes). As a first step to reach the pregnant uterus, PRV has to cross the endothelial cell barrier of the maternal blood vessels. The current aim was to investigate *in vitro* whether immune-masked PRV-infected monocytes can transmit PRV in the presence of virus-neutralizing antibodies via adhesion and fusion of these monocytes with endothelial cells. Porcine blood monocytes, infected with a LacZ-carrying PRV strain, were incubated with PRV-specific antibodies to induce internalization. Then, cells were co-cultivated with endothelial cells for different periods of time. Only PRV-infected monocytes with internalized viral cell surface proteins efficiently adhered to endothelial cells. LacZ transmission to endothelial cells, as a measure for monocyte-endothelial cell fusion, could be detected after co-cultivation from 30 min onwards. Viral transmission was confirmed by the appearance of plaques. Adhesion of immune-masked PRV-infected monocytes to endothelial cells was mediated by the cellular adhesion molecules wCD11R3 and CD18 and subsequent fusion was mediated by the virus. In conclusion, immune-masked PRV-infected monocytes can adhere and subsequently transmit virus to endothelial cells in the presence of virus-neutralizing antibodies.

Introduction

Pseudorabies virus (PRV) is a member of the *Alphaherpesvirinae* and causes Aujeszky's disease in its natural host, the pig. Clinical signs depend mostly on the virulence of the virus strain and the age of the affected pig (Kluge *et al.*, 1992). Infection with a virulent strain is characterized by nervous signs in newborn pigs, respiratory disorders in fattening pigs and reproductive failure in sows (Wittmann *et al.*, 1980). In the presence of a vaccination-induced immunity, PRV still can replicate, resulting in a restricted viraemia (Wittmann *et al.*, 1980). In general, such a viraemia does not cause problems; however, abortion may still occur as a result of cell-mediated transplacental spread and intrafetal replication (Nauwynck & Pensaert, 1992). Porcine blood monocytes have been shown to be essential to transport the virus to the pregnant uterus in vaccination-immune pigs (Nauwynck & Pensaert, 1992; Nauwynck & Pensaert, 1995a). Exactly how these infected monocytes survive in the blood in the presence of an activated immunity and subsequently transmit the virus to endothelial cells of the placental blood vessels, as a first step to reach the fetuses, is still poorly understood.

PRV-infected monocytes express viral envelope proteins in their plasma membrane (Favoreel *et al.*, 1999). Antibodies bind to these viral glycoproteins which should induce antibody-dependent lysis of the infected cells (Sissons & Oldstone, 1980). Apparently, this does not happen efficiently in PRV-infected monocytes. Earlier, it has been shown that addition of PRV-specific antibodies to PRV-infected monocytes results in aggregation of the membrane-bound viral glycoproteins, followed by the internalization of these glycoprotein-antibody aggregates. This antibody-dependent internalization is fast and efficient, is mediated by the viral proteins gB and gD (Favoreel *et al.*, 1999,2002; Van de Walle *et al.*, 2001) and protects infected monocytes from efficient antibody-dependent cell lysis *in vitro* (Van de Walle *et al.*, 2003). This mechanism may help to explain how infected monocytes survive in the blood of vaccinated animals. How these infected monocytes with internalized viral cell surface proteins (immune-masked monocytes) are then able to transmit PRV to the fetuses in the presence of neutralizing antibodies remains unclear. As a first step to reach the fetuses, PRV has to cross the endothelial cell barrier of the maternal blood vessels. A likely scenario how this happens is that PRV-infected monocytes

adhere to the endothelial cells and, subsequently, PRV crosses the endothelial barrier in the presence of virus-neutralizing antibodies. At least two hypotheses as to how the latter occurs can be put forward: on the one hand, immune-masked PRV-infected monocytes may cross the endothelial cell barrier by means of diapedesis; on the other hand, immune-masked PRV-infected monocytes may fuse with the vascular endothelial cells, thereby transmitting virus to these cells. In both cases, transmission of virus to endothelial cells is the very first step required for PRV to reach the fetuses. Although the subsequent steps are still to be unraveled, it has been shown before that all these steps can occur through direct cell-to-cell spread of the virus. Indeed, monocytes infected with a gD_{null} mutant, which can only spread through direct cell-to-cell transport (Rauh & Mettenleiter, 1991; Peeters *et al.*, 1992a), still can induce abortion in vaccinated sows (Nauwynck, 1997).

Diapedesis of leukocytes at sites of inflammation is a well-known process (reviewed by Muller & Randolph, 1999) and in general requires the action of chemokines (Baggiolini, 1998). The hypothesis of diapedesis as a means of PRV transmission through the endothelial barrier can certainly not be ruled out as a possible means of PRV transmission to fetal tissues and may be worth investigating in more detail. However, in the case of PRV-induced abortion, there are no indications of inflammation or local production of chemokines at the placental site or other triggers that could induce diapedesis of PRV-infected monocytes. Therefore, the aim of the current study was to evaluate the second hypothesis, comprising the adhesion and subsequent fusion of immune-masked PRV-infected monocytes with endothelial cells. In this context, it is noteworthy that contact between infected macrophages or neutrophils and endothelial cells has been suggested to be implicated in the dissemination of human cytomegalovirus (HCMV) from and to endothelial cells (Waldman *et al.*, 1995; Grundy *et al.*, 1998). Spread of HCMV to the embryo or fetus is well documented and is probably mediated by infected trophoblasts, macrophages and endothelial cells (Fisher *et al.*, 2000).

Alphaherpesviruses are well-known for their cell-to-cell spread and the roles of different viral glycoproteins in PRV cell-to-cell spread have been extensively studied by the use of PRV deletion mutants. Viral glycoproteins gB and gH/gL have been shown to be essential for cell-to-cell spread, whereas gE and gD are modulatory (Klupp et al., 1997; Peeters *et al.*, 1992a; Peeters *et al.*, 1992b; Rauh & Mettenleiter, 1991; Zsak *et al.*, 1992). Recently, it was suggested that the betaherpesvirus HCMV

spreads from infected endothelial cells to leukocytes via microfusion events (Gerna *et al.*, 2000).

The aim of the present study was to investigate *in vitro* if immune-masked PRV-infected monocytes can transmit PRV to endothelial cells via adhesion and fusion in the presence of virus-neutralizing antibodies and if so, what the underlying mechanism is.

Materials and Methods

Virus strains. The PRV Kaplan mutants ΔgG, ΔgD, ΔgB and ΔgH were used. All these strains carry the ß-galactosidase gene (LacZ) as described earlier (Mettenleiter & Rauh, 1990; Rauh & Mettenleiter, 1991; Rauh et al., 1991; Babic et al., 1996).

Antibodies. Unlabeled and FITC-labeled protein A-purified polyclonal IgG antibodies were used, derived from PRV (89V87) inoculated pigs, originating from a PRV-negative farm (Nauwynck *et al.*, 1995b). Unlabeled anti-PRV IgG antibodies with a titer of 512, as determined by a serum neutralization test (Andries *et al.*, 1978), were used in the culture medium to inhibit virus transmission via extracellular medium. The FITC-labeled anti-PRV IgG antibodies were used to induce internalization of viral cell surface proteins in PRV-infected monocytes, at a concentration of 0.33 mg/ml (Favoreel *et al.*, 1999). To visualize the cellular adhesion molecules CD15, CD11a, wCD11R3 and CD18 on porcine monocytes, monoclonal antibodies DU-HL60-3 (Sigma; Whyte & Binns, 1994), BL1H8, 2F4/11 and BA3H2 (Alvarez *et al.*, 2000; Dominguez *et al.*, 2001) respectively, were used.

Isolation of porcine arterial endothelial cells. Endothelial cells were isolated as described previously by Nauwynck and Pensaert (1995b), with some modifications. Briefly, endothelial cells were obtained from the *aorta* of 5 to 6 week old piglets by flushing several times with 2.5 mg/ml trypsin (Sigma Chemical Company, St. Louis, Missouri, USA) at 37°C. The collected cell fractions were centrifuged for 10min at 260xg and resuspended in endothelial growth medium, based on Dulbecco's MEM® (Gibco BRL, Life Technologies Inc., Paisley, Scotland) and supplemented with 10% fetal bovine serum (FBS), 0.6 mg/ml glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.1 mg/ml kanamycin, 1 mM sodium pyruvate and 1% non-essential amino acids 100x (Gibco BRL). Cells were seeded on Corning cell culture flasks (Costar Corporation, Cambridge, USA). Purity of endothelial cells was analysed by fluorescence staining with 1,1'-dioctadecyl-1-3,3,3',3'-tetramethylindocarbocyanine-perchlorate-acetylated low density lipoprotein (DiI-Ac-LDL) (Biomedical Technologies Inc., Stoughton, USA) (Voyta *et al.*, 1984) and was always ≥ 90%. Cells from the first to third passages were harvested by trypsinization, seeded on 4-

well multidish (Nunc A/S, Roskilde, Denmark) in endothelial growth medium and used upon confluency.

Isolation of porcine blood monocytes. PRV-negative pigs were used as blood donors. Blood was collected from the *vena jugularis* on heparin (15U/ml) (Leo, Zaventem, Belgium). Blood mononuclear cells were separated on Ficoll Paque® (Amersham Pharmacia Biotech AB, Uppsala, Sweden) following the manufacturer's instructions. Mononuclear cells were then resuspended in medium (A), based on RPMI-1640® (Gibco BRL, Life Technologies Inc., Paisley, Scotland) and supplemented with 10% FBS, 0.3 mg/ml glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.1 mg/ml kanamycin, 1 mM sodium pyruvate, 1% non-essential amino acids 100x (Gibco BRL) and 10 U/ml heparin. Afterwards, cells were seeded on 58 mm petri dishes with cell culture coating (Nunc A/S, Roskilde, Denmark) at a concentration of $2x10^6$ cells/ml and cultivated at 37°C with 5% CO_2. After 48 h, non-adhering lymphocytes were removed by washing the petri dishes three times with RPMI-1640.

Inoculation of blood monocytes. After the removal of lymphocytes, adherent cells, consisting of ≥ 70% of monocytes, as assessed by flow cytometric analysis after indirect immunofluorescence staining with the monocyte marker 74.22.15 (Pescovitz *et al.*), were inoculated with the different PRV strains at a multiplicity of infection (m.o.i.) of 20 in 0.5 ml medium (A) without heparin. Cells were further incubated at 37°C with 5% CO_2. For all strains used and for all experiments, between 80 and 90% of the monocytes were infected.

Incubation of PRV-infected monocytes with porcine anti-PRV polyclonal antibodies (pAbs). At 13 h post inoculation (p.i.) with PRV, monocytes were centrifuged for 10min at 500xg, washed and resuspended in medium (A). Cells were incubated for 1 h at 37°C with FITC-labeled PRV pAbs (as described by Favoreel et al., 1999). Cells were washed two times with RPMI-1640® and then used in the adhesion/fusion assay. As a negative control, 50 µg/ml of genistein (Sigma Chemical Company, St. Louis, Missouri, USA) was added 45 min before and also during antibody incubation. Genistein inhibits tyrosine kinase activity and therefore

antibody-induced internalization of viral proteins (Favoreel et al., 1999). This concentration of genistein had no effect on cell viability as determined earlier (Favoreel et al., 1999) and had also no effect on adhesion or fusion processes (data not shown).

Definition of the viral protein distribution. The viral protein distribution was scored as 'no internalization' when the fluorescence label exhibited a homogeneous or patched cell surface cover. The viral glycoproteins were considered 'internalized' when viral proteins were located in vesicles inside the cell.

Adhesion/fusion assay. After incubation with FITC-labeled PRV pAbs in the presence or absence of genistein (as described above), PRV-infected monocytes were washed, counted and resuspended in endothelial growth medium, supplemented with PRV-neutralizing IgG antibodies and 50 μg/ml genistein. To each well of endothelial cells, approximately $1.5x10^5$ monocytes were added, followed by centrifugation for 2 min at 44xg. At different time points of co-cultivation (0, 30, 60 and 120 min), wells were washed two times thoroughly with endothelial growth medium and fixed for 10 min with 2% formaldehyde and 0.2% glutaraldehyde (Merck & Co., Inc., West Chester, USA). The 4-well multidishes, consisting of monocytes and endothelial cells, were then stained for ß-galactosidase activity with X-gal, following the manufacturer's instructions (Invitrogen LTD., Paisley, Scotland). After 2 hours, cells were mounted in a glycerin-phosphate buffered saline solution (PBS) (0.9:0.1, vol/vol) with 2.5% 1,4-diazabicyclo(2.2.2)octane (DABCO; Janssen Chimica, Beerse, Belgium). Quantitative results were obtained by examining the X-gal signal (showing adhesion/fusion) by light microscopy and the fluorescence distribution (showing internalization/no internalization) by fluorescence microscopy. Cells were excited with an Osram HBO 50-W bulb using a I3 filter and observed with a Leitz DM RBE microscope (Wild Leitz GmbH, Heidelberg, Germany). Images were obtained by using a Sony color video camera (model DXC-9100P), linked to a Macintosh computer.

The percentage of adhesion of monocytes to endothelial cells was calculated as follows: (number of adhered monocytes at time point x / number of monocytes added per well) × 100. To determine the percentage of monocytes fused with endothelial

cells, the following formula was used: (number of LacZ positive endothelial cells at time point x / number of adhered monocytes at time point x) × 100. All assays were run independently at least three times.

Some wells were, instead of fixed, further cultivated at 37°C with 5% CO_2 in the presence of virus-neutralizing antibodies and controlled for plaque formation at 30 h of co-cultivation.

Indirect immunofluorescence staining of cellular adhesion proteins on the cell surface of PRV-infected monocytes before and after antibody-induced internalization of viral cell surface proteins. Monocytes (isolated as described above) were seeded on 4-well chambered coverglasses (Nunc A/S, Roskilde, Denmark) at a concentration of $2.5x10^6$ cells/ml and inoculated with PRV. Monocytes, at 13 h p.i. with PRV, were incubated with 0.33 mg/ml FITC-labeled PRV pAbs and fixed for 10 min with 3% formaldehyde at different time points (0 min, before internalization; 60 min, after internalization). Cells were washed two times with RPMI-1640® and incubated for 1 h at 37°C with α-CD15 (dilution 1:50), α-CD11a (dilution 1:10), α-wCD11R3 (dilution 1:30) or α-CD18 (dilution 1:30). Cells were washed again two times with RPMI-1640® and subsequently incubated for 1 h at 37°C with goat anti-mouse-Texas Red (dilution 1:50) (Molecular Probes, Eugene, Oregon). Finally, cells were washed thoroughly, mounted in a glycerin-PBS solution and analyzed by confocal microscopy.

Adhesion-blocking assay. Experiments were performed as described for the adhesion/fusion assay, with some modifications. After incubation for 1 h at 37°C with FITC-labeled PRV pAbs in the absence of genistein (to allow internalization of viral cell surface proteins), PRV-infected monocytes were washed and subsequently incubated for 30 min at 37°C with medium A, supplemented with the monoclonal antibodies α-CD11a, α-wCD11R3 and/or α-CD18 (dilution 1:10). After washing and counting, monocytes were added to wells of endothelial cell, fixed at 0, 30 and 60 min of cocultivation, and stained for ß-galactosidase activity with X-gal (as described above). As a control, no antibodies were supplemented to medium A.

Confocal Laser Scanning Microscopy. Double stained samples of viral proteins and cellular adhesion molecules were examined with a Leica TCS SP2 laser scanning spectral confocal system (Leica Microsystems GmbH, Wetzlar, Germany) and linked to a DM IRB inverted microscope (Leica Microsystems). Argon and HeNe laser lights were used to excite FITC (488 nm line) and Texas red (543 nm line) fluorochromes. Extended focus images were obtained with Leica confocal software.

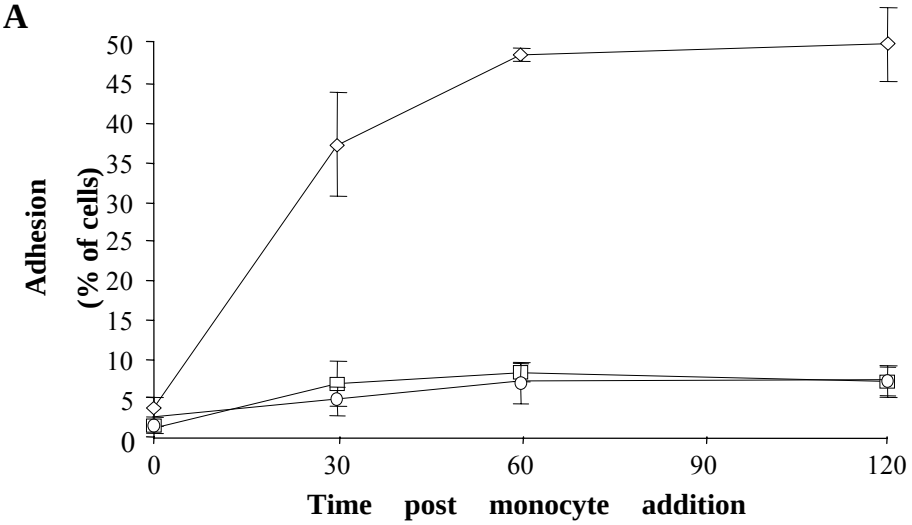
Results

Internalization of viral cell surface proteins in PRV-infected monocytes (immune-masked monocytes). To examine the possible adhesion and fusion of immune-masked PRV-infected monocytes with endothelial cells, porcine monocytes were inoculated with a PRV strain carrying the β-galactosidase fusion gene LacZ. Transmission of the enzyme β-galactosidase (which can be visualized easily by adding the substrate X-gal) from the PRV-infected monocyte to endothelial cells in the presence of PRV-neutralizing antibodies was used as a measure of fusion. A PRV strain carrying the LacZ gene inserted in the gG locus (Kaplan ΔgG) was chosen, since this mutant has been shown to exhibit wild type growth properties (Mettenleiter & Rauh, 1990). Moreover, this mutant has also been shown to have no effect on the efficiency of antibody-induced internalization of viral glycoproteins in PRV-infected monocytes (Favoreel *et al.*, 1999).

Adhesion of immune-masked PRV-infected monocyte to endothelial cells. PRV Kaplan ΔgG-infected monocytes, at 13 h p.i., were incubated for 1 h at 37°C with FITC-labeled PRV pAbs as described in the Material and Methods section. This antibody incubation resulted in 63.5% ± 2.5 of monocytes with internalized viral cell surface proteins (immune-masked monocytes) and 36.5% ± 1.2 of cells without internalized viral cell surface proteins (for images of cells without internalized viral cell surface proteins see Fig. 3.A; for images of cells with internalized viral cell surface proteins see Fig. 3.B). The mixture of monocytes with and without internalized viral cell surface proteins were then added to a monolayer of endothelial cells at 37°C in the presence of virus-neutralizing antibodies and adhesion kinetics of both types of monocytes (with and without internalized viral glycoproteins) were determined. Fig. 1.A shows (i) that adhesion of monocytes to endothelial cells reaches its plateau at 60 min of co-cultivation and (ii) that the immune-masked PRV-infected monocytes (with internalized viral cell surface proteins) adhere much more efficiently to the endothelial cells than PRV-infected monocytes without internalization. To confirm the latter, experiments were repeated in the presence of genistein, a reagent known to inhibit antibody-induced internalization (Favoreel *et al.*, 1999). Adding genistein before and during antibody addition (as described in Materials and Methods)

resulted in a population of 94.3% ± 1.5 of PRV-infected monocytes without internalization. Adhesion efficiency of these monocytes to endothelial cells was low (Fig.1.A).

A



B

	% of PRV-infected monocytes with internalized viral cell surface proteins	% of PRV-infected monocytes adhered to endothelial cells
ΔgG reference	62.4 ± 1.5	39.4 ± 4.3
ΔgB mutant	36.0 ± 2.6 *	20.0 ± 4.5 *
ΔgD mutant	38.0 ± 1.7 *	23.0 ± 6.5 *

FIG. 1. (A). Kinetics of adhesion of PRV-infected monocytes with endothelial cells in the presence of virus neutralizing antibodies. Monocytes were harvested at 13 h p.i. and incubated for 1 h at 37°C with FITC labeled PRV-specific antibodies and added to endothelial cells. Graphs represent kinetics for monocytes with internalized viral cell surface proteins (=immune-masked monocyte) (◊) or without internalized viral cell surface proteins (□), or treated with genistein (O). The data are means ± standard deviations of triplicate assays.

(B). Percentage of internalization (at 1 h post antibody addition) and adhesion (at 60 min of cocultivation) of monocytes, inoculated for 13 h with the PRV Kaplan ΔgG reference strain, ΔgB mutant or ΔgD mutant. Data represent means ± standard deviations of triplicate assays. Asterisks indicate significant differences ($p < 0.01$).

It was reported previously that monocytes infected with the PRV Kaplan mutants ΔgB and ΔgD have reduced antibody-induced internalization efficiencies (Favoreel *et*

al., 1999; Van de Walle *et al.*, 2001). Monocytes inoculated with these strains for 13 h and incubated with PRV pAbs for 1 h, showed reduced adhesion efficiencies compared to the reference strain (Fig. 1.B). Reduction in adhesion efficiency of the ΔgB and ΔgD mutants compared to the wild type virus corresponded fairly close to their reduction in internalization efficiency, suggesting further that, in the presence of neutralizing antibodies, internalization of viral cell surface proteins correlates directly with an increase in adhesion efficiency of infected monocytes to endothelial cells. Taken together, these results show that efficient internalization of antigen-antibody complexes from the cell surface of infected monocytes is essential to allow efficient adhesion of PRV-infected monocytes to endothelial cells in the presence of virus-neutralizing PRV pAbs.

Fusion of the immune-masked PRV-infected monocytes with endothelial cells. Fig. 2.A shows that, following adhesion, immune-masked PRV-infected monocytes fused with underlying endothelial cells in the presence of neutralizing antibodies. This was demonstrated by β-galactosidase transmission from the cytoplasm of the infected monocyte to the underlying endothelial cells (Fig. 2.A). Fig. 2.B shows that fusion of the immune-masked PRV-infected monocytes started from 30 min of co-cultivation, reaching a level of 38.0% ± 2.5 of fused immune-masked monocytes at 120 min of co-cultivation (end of the experiment). The presence of β-galactosidase in the endothelial cells was caused by fusion between infected monocytes and endothelial cells and not by infection of the endothelial cells by cell-free virus, as endothelial cells, infected with cell-free LacZ-carrying ΔgG PRV (in the absence of neutralizing antibodies), only became β-galactosidase positive from 3 h p.i. onwards (data not shown).

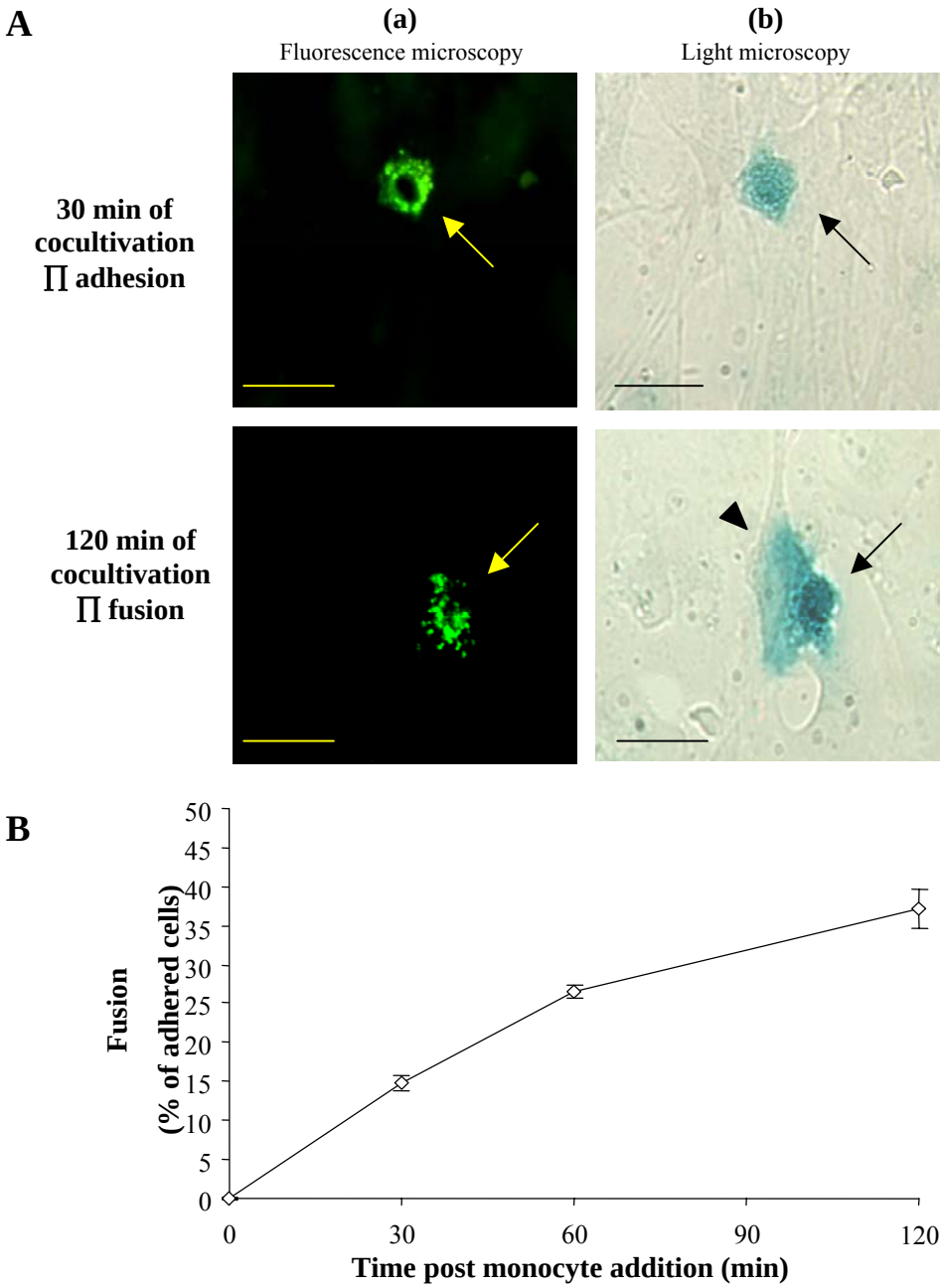


FIG. 2. (A). Double labeling of viral cell surface proteins (FITC) (a) and β-galactosidase activity (X-gal) (b) of PRV-infected monocytes (arrow), at 30 min (lane 1) and 120 min (lane 2) of cocultivation. Lane 1 shows a PRV-infected monocyte with internalized viral cell surface proteins (arrow), adhered to the underlying endothelial cells. Lane 2 shows a PRV-infected monocyte with internalized viral cell surface proteins (arrow) fused with the underlying endothelial cells (endothelial cells stain positive for β-galactosidase activity, arrowhead). Bar 15µm. (B). Kinetics of fusion of PRV-infected monocytes with internalized viral cell surface proteins (= immune-masked monocytes) with underlying endothelial cells. Data represent means ± standard deviations of triplicate assays.

Viral transmission from the immune-masked monocyte to endothelial cells, in the presence of virus-neutralizing antibodies, was confirmed by the appearance of plaques at 30 h of co-cultivation (Fig. 2.C). the number of plaques was similar to the number to be expected based on the percentage of fused monocytes.

C

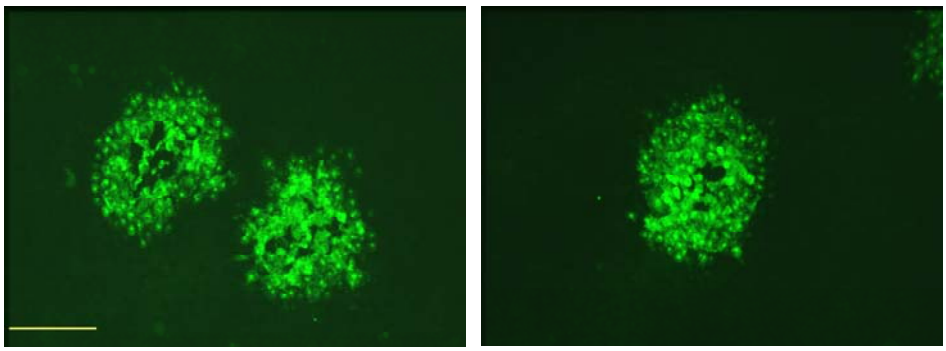


FIG. 2. (C) Fluorescence microscopy images of plaques in a monolayer of endothelial cells at 30 h of cocultivation with PRV-infected monocytes in the presence of virus-neutralizing antibodies, fixed in 100% methanol and stained for viral antigens using FITC-labeled PRV-specific antibodies. Bar 0.5mm.

Adhesion is mediated by cellular adhesion molecules. Antibody-induced internalization of PRV-infected monocytes results in infected monocytes with no or only very few viral protein-antibody complexes on their surface (Favoreel *et al.*, 1999). This implies that viral protein(s) most likely are not responsible for the adhesion to endothelial cells and that the adhesion occurs via another (cellular) mechanism. Cellular adhesion processes of monocytes to endothelial cells are well studied. They are mediated by different adhesion molecules on the cell surface of the monocytes such as Sialyl Lewis^x (also designated CD15), the β1- and β2-integrins (Tedder *et al.*, 1995; Stewart *et al.*, 1995; Bullido *et al.*, 1996). However, it has been shown before that internalization of viral cell surface proteins in PRV-infected monocytes, induced by PRV-specific antibodies, results in co-internalization of several, uncharacterized, cellular proteins (Favoreel *et al.*, 1999). Therefore, we first investigated if and which adhesion molecules are still present on the plasma membrane of PRV-infected monocytes after the antibody-induced internalization process.

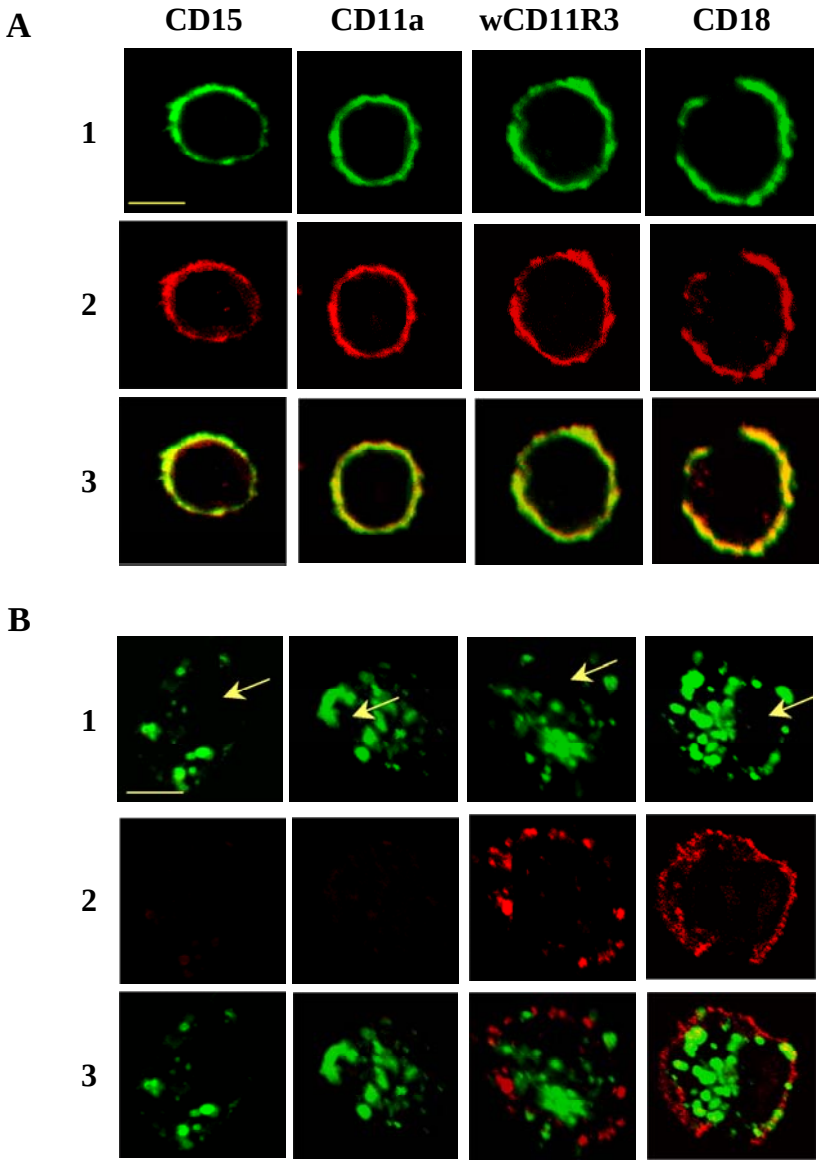


FIG. 3. (A) & (B). Double immunofluorescence labeling of viral cell surface proteins (FITC) with different cellular adhesion molecules (CD15, CD11a, wCD11R3 and CD18) (Texas red) before (A) and after (B) antibody-induced internalization of viral cell surface proteins. Monocytes, at 13h p.i. with PRV, were incubated for 1 h at 37°C with FITC-labeled PRV-specific antibodies. At time points 0 min (A) and 60 min (B) post antibody addition, cells were fixed for 10min with 3% formaldehyde. Afterwards, cells were incubated for 1h at 37°C with monoclonal antibodies against different cellular adhesion molecules, followed by incubation for 1 h at 37°C with goat anti-mouse Texas red. Lane 1 shows the viral cell surface proteins, lane 2 shows the cellular adhesion molecules, and lane 3 shows images obtained by merging (1) and (2). The images are middle sections of each cell. Arrow indicates the position of the nucleus. Bar 5 μm.

Double immunofluorescence staining of CD15 and some β2-integrins, known to be present on porcine monocytes on the one hand and viral cell surface proteins on the other hand, demonstrated that the adhesion molecules CD15, CD11a, wCD11R3 and CD18 were present on the plasma membrane of PRV-infected monocytes before the antibody-induced internalization process (Fig. 3.A). After antibody-induced

internalization of viral cell surface proteins on the infected monocytes, however, only the adhesion molecules wCD11R3 and CD18 were still visually present on the plasma membrane (Fig. 3.B). Hence, wCD11R3 and CD18 may possibly play a role during adhesion of the immune-masked monocytes to endothelial cells. To examine this, an adhesion blocking assay was performed using monoclonal antibodies directed against these adhesion molecules. Fig. 3.C shows that pre-incubating immune-masked PRV-infected monocytes with α-wCD11R3, α-CD18 or α-wCD11R3 + α-CD18, all resulted in a significant decrease of the percentage of PRV-infected monocytes adhered to endothelial cells ($p < 0.005$, one way anova), compared to non-treated cells.

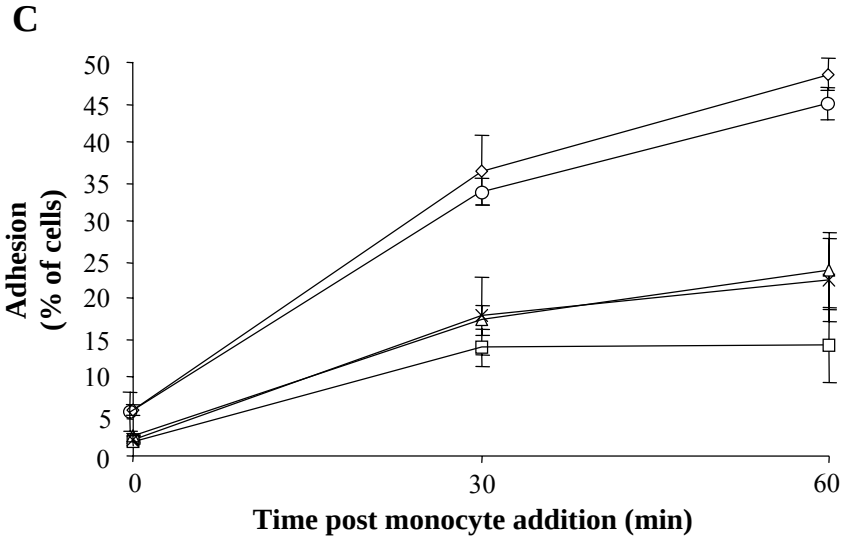


FIG. 3. (C). Adhesion blocking assay. Kinetics of adhesion of immune-masked PRV-infected monocytes, pre-incubated with a mixture of α-wCD11R3 and α-CD18 (□), with α-wCD11R3 (Δ), with α-CD18 (Σ) or with α-CD11a (O), to endothelial cells. As a control, non-treated PRV-infected monocytes (◊) were used. Data represent means ± standard deviations of triplicate assays.

Pre-incubating the immune-masked PRV-infected monocytes with α-CD11a, an adhesion molecule no longer present on the plasma membrane of PRV-infected monocytes after antibody-induced internalization, resulted in adhesion efficiencies comparable to non-treated cells (Fig. 3.C). Hence, wCD11R3 and CD18 are important during adhesion of the immune-masked PRV-infected monocytes to endothelial cells.

Fusion is viral mediated. To investigate if fusion of immune-masked PRV-infected monocytes with endothelial cells is mediated by viral proteins, we inoculated monocytes with the PRV mutant ΔgH. This PRV mutant (i) carries the ß-galactosidase fusion gene (LacZ) (Babic *et al.*, 1996), (ii) is known to be essential for virus-cell fusion and cell-to-cell spread of PRV (Peeters *et al.*, 1992b) and (iii) is not

important for the antibody-induced internalization process (Favoreel *et al.*, 1999; Van de Walle *et al.*, 2001). Since PRV ΔgH has no effect on efficient antibody-induced internalization, using PRV ΔgH had no effect on the percentage of adhesion of PRV-infected monocytes to endothelial cells compared to the ΔgG reference strain (Fig. 4.A). Fig. 4.B shows that ΔgH-infected immune-masked monocytes are unable to fuse with endothelial cells ($p < 0.01$, one way ANOVA). This implies that fusion is viral-mediated, probably by a mechanism similar to virus-cell fusion.

A

	% of PRV-infected monocytes with internalized viral cell surface proteins	% of PRV-infected monocytes adhered to endothelial cells
ΔgG reference	64.0 ± 1.9	34.4 ± 1.9
ΔgH mutant	60.3 ± 0.8	33.9 ± 2.6

B

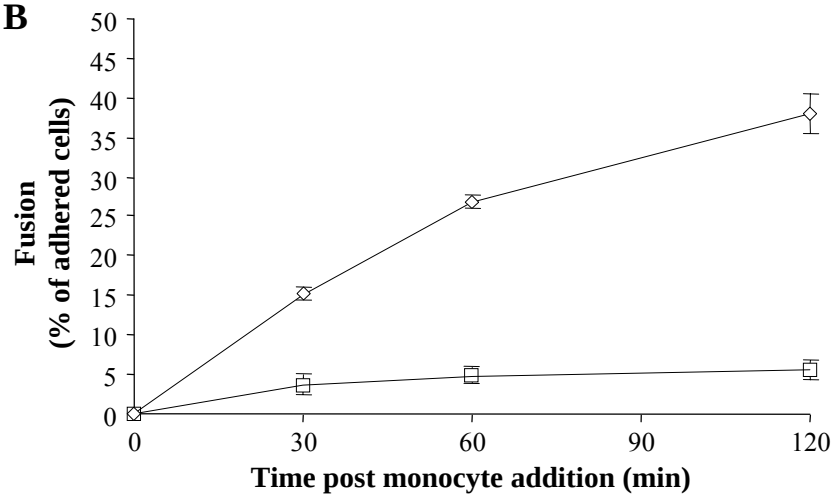


FIG. 4. (A). Percentage of internalization of viral cell surface proteins (at 1 h post antibody addition) and adhesion (at 60 min of cocultivation with endothelial cells) of monocytes, inoculated for 13 h with the PRV Kaplan ΔgG reference strain or ΔgH mutant. Data represent means ± standard deviations of triplicate assays. (B). Kinetics of fusion of immune-masked monocytes, infected with the PRV Kaplan ΔgG reference strain (◊) or ΔgH mutant (□) with endothelial cells. Data represent means ± standard deviations of triplicate assays.

Discussion

Pseudorabies virus (PRV)-infected porcine blood monocytes have been shown to transport the virus to different internal organs of vaccinated pigs, in the presence of PRV-neutralizing antibodies (Wittmann *et al.*, 1980; Nauwynck & Pensaert, 1992, 1995a). However, infected monocytes express viral proteins in their plasma membrane (Favoreel *et al.*, 1999) and binding of PRV-specific antibodies to these viral proteins should therefore induce antibody-dependent lysis of the infected cells. Recently, a mechanism has been postulated how PRV-infected monocytes potentially may avoid efficient antibody-dependent lysis: binding of PRV-specific antibodies to the viral cell surface proteins has been shown to activate a signal to the cell, mediated by viral proteins gB and gD, followed by rapid internalization of the majority of antigen-antibody complexes present on the cell surface (Favoreel *et al.*, 1999, 2002). This mechanism protects PRV-infected monocytes with internalized viral cell surface proteins (immune-masked monocytes) from efficient complement-mediated lysis *in vitro* (Van de Walle *et al.*, 2003).

In order for PRV to be able to reach the pregnant uterus in vaccinated animals, these immune-masked PRV-infected blood monocytes should be capable of transmitting the virus, in the presence of neutralizing antibodies, to the endothelial cells of the placental blood vessel walls. Here, we showed in an *in vitro* assay that in the presence of virus-neutralizing antibodies, immune-masked monocytes can efficiently adhere to the endothelial cells, followed by transmission of virus through fusion of the monocyte with the endothelial cells.

PRV-infected monocytes with internalized viral cell surface proteins (immune-masked monocytes) adhered much more efficiently to endothelial cells than PRV-infected monocytes without internalization. These results indicate that, in the presence of virus-neutralizing antibodies, antibody-induced internalization of viral cell surface proteins is important for efficient adhesion of PRV-infected monocytes to endothelial cells. Why are PRV-infected cells without internalized viral cell surface proteins unable to adhere efficiently to the endothelial monolayer? PRV-infected monocytes without internalized viral cell surface proteins, in contrast to immune-masked monocytes, are covered by PRV-specific antibodies, which possibly may result in (sterically) hindered access of the adhesion molecules on the monocytes (cellular as

well as viral such as gC and gD) to adhesion receptors on the underlying endothelial cells. This is consistent with earlier results showing that PRV-infected cells, covered with PRV-neutralizing antibodies, are unable to adhere to non-infected cells (Hanssens *et al.*, 1993).

Since monocytes with internalized viral cell surface proteins possess only very few, if any, viral proteins on their plasma membrane (Favoreel *et al.*, 1999), adhesion of immune-masked monocytes to endothelial cells is most likely mediated by cellular, and not viral, adhesion molecules on the cell surface. Physiological adhesion requires the interaction of integrins, such as CD11a/CD18 (also designated LFA-1) and CD11b/CD18 (also designated MAC-1) on monocytes with the Ig superfamily (ICAM-1, ICAM-2 and VCAM-1) on endothelial cells (Stewart *et al.*, 1995; Bullido *et al.*, 1996). However, it has been shown before that during internalization of viral cell surface proteins induced by PRV-specific antibodies, at least certain, unidentified, cellular proteins undergo co-internalization with the cell surface proteins (Favoreel *et al.*, 1999). Determining if and which cellular adhesion molecules remain on the plasma membrane of the immune-masked PRV-infected monocytes was therefore essential as a first step to examine the process of adhesion of the immune-masked monocytes to endothelial cells. Double immunofluorescence stainings before and after the antibody-induced internalization of viral cell surface proteins showed that significant amounts of the adhesion molecules wCD11R3 and CD18 remained on the plasma membrane after antibody-induced internalization, whereas CD15 and CD11a were no longer visually present on the cell surface, suggesting that the adhesion molecules wCD11R3 and CD18, but not CD15 and CD11a, may be involved in the adhesion of the immune-masked monocytes to endothelial cells. WCD11R3 and CD18 were confirmed to be involved in the adhesion process, since pre-incubation of the immune-masked PRV-infected monocytes with monoclonal antibodies against these adhesion molecules, but not against CD11a, significantly reduced adhesion. Similarly, the CD18 molecule has been shown to be involved in the adhesion of peripheral blood leukocytes to HCMV-infected endothelial cells (Span *et al.*, 1991). The reduction of adhesion of the immune-masked PRV-infected monocytes after pre-incubation with monoclonal antibodies against wCD11R3 and CD18 was, although significant, only approximately 65%, which may imply the involvement of other adhesion molecules. Besides CD11a and wCD11R3, there exist two other CD11 molecules ,wCD11R1 and wCD11R2, which may be involved during adhesion

(Dominguez *et al.*, 2001). Further, for human monocytes it has been described that adhesion to endothelial cells can also occur via the β1-integrin VLA-4, although to a much lesser extent than via the β2-integrins CD11/CD18 (Meerschaert & Furie, 1994). The expression of VLA-4 has also been described for porcine leukocytes, although its role in adhesion to porcine endothelial cells has, to our knowledge, not yet been investigated (Whyte & Binns, 1994). A possible hypothesis for the very efficient adhesion via cellular adhesion molecules could be that PRV induces an upregulation of certain adhesion molecules upon infection, as has already been described for many viruses, including herpesviruses. For example, for herpes simplex virus type 1, it has been shown that virus-infected blood monocytes adhere much more efficiently to human endothelial cells compared to non-infected monocytes, due to the production of certain cytokines, leading to an upregulation of cellular adhesion molecules on endothelial cells (Larcher *et al.*, 2001). Further, HMCV has been shown to induce an upregulation of adhesion molecules in infected endothelial cells resulting in an increased adhesion of the endothelial cells to peripheral blood leukocytes (Shahgasempour *et al*., 1997). Based on the present study, no indications were found for a similar upregulation of adhesion molecules in PRV-infected monocytes. However, the potential of such a putative upregulation to increase the chances of PRV to cross the barrier of endothelial cells may make it worth investigating in more detail.

Following adhesion, immune-masked monocytes fuse with endothelial cells. The rate of fusion was lower than the rate of adhesion, possibly suggesting that upon adhesion, expression/transport of viral proteins (e.g. gB, gH/gL) to the cell surface was necessary to induce fusion. Indeed, fusion was shown to be virus mediated, since inoculating monocytes with a LacZ-carrying ΔgH mutant abolished the fusion capacity of the immune-masked monocytes (without affecting internalization and adhesion efficiencies). Fusion was shown to result in virus transmission to endothelial cells by the appearance of plaques. Only about 50% of the adhered monocytes fused with the endothelial cells, even after 240 min of co-cultivation of monocytes with endothelial cells (data not shown), indicating that a significant fraction of immune-masked monocytes are incapable of fusing with endothelial cells. The reason for this inability to fuse is speculative but one explanation could be that, upon adhesion to the endothelial cells, not all monocytes are triggered efficiently to resume expression of viral proteins on the cell surface.

Adhered leukocytes, either during immune cell-to-cell contact or during contact with epithelial/endothelial cells, are generally polarized (Sanchez-Madrid & del Pozo, 1999; Bromley *et al.*, 2001). Such polarization may support efficient virus spread, since it may result in predominant transport of viral cell surface proteins (implicated in cell fusion) or even virions, to the contact area between both cells. Such unidirectional deposition virions at the contact site between infected leukocytes and contact cells has already been described for human immunodeficiency virus (Pearce-Pratt *et al.*, 1994; Fais *et al.*, 1995) and may be worth investigating during the interaction of immune-masked PRV-infected monocytes with endothelial cells.

In conclusion it can be stated that immune-masked PRV-infected monocytes efficiently adhere to and subsequently fuse with endothelial cells in the presence of virus-neutralizing antibodies *in vitro*. The adhesion process is mediated by cellular adhesion molecules wCD11R3 and CD18 and subsequent fusion is mediated by the virus. This adhesion and fusion process gives a potential explanation as to how PRV-infected monocytes transmit virus to vascular endothelial cells in vaccinated animals, as a first step to reach internal organs such as the pregnant uterus.

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6.

GENERAL DISCUSSION

General Discussion

Despite the presence of an immunity, induced by inactivated vaccines, a cell-associated viraemia of pseudorabies virus (PRV) can still occur, enabling the virus to reach internal organs and occasionally, to cause abortion (Wittmann *et al.*, 1980; de Mûelenaere & Pensaert, 1989). Porcine blood monocytes are essential to transport the virus throughout the body of these vaccinated animals, in the presence of virus-specific antibodies (Nauwynck & Pensaert, 1992). This virus transport in the presence of virus-specific antibodies indicates that some PRV-infected monocytes are able to survive in the blood long enough to transmit the virus to internal organs, including the pregnant uterus. This implies that recognition and destruction of the virus-infected cells by antibody-dependent cell lysis occurs inefficiently. One of the mechanisms that may aid PRV in masking infected blood monocytes from the antibody-dependent cell lysis consists of the antibody-induced internalization of antigen-antibody complexes that are present on the cell surface of infected monocytes (Favoreel *et al.*, 1999). Using *in vitro* models, it was the goal of this thesis to gain further insight in how this process of antibody-induced internalization may help PRV-infected monocytes to reach fetal tissues in the presence of antibody-dependent components of the immune system. The overall hypothetical model illustrating how PRV may reach internal organs in vaccinated animals, based on the *in vitro* findings in this thesis, is presented in the scheme at the end of the General Discussion.

We demonstrated *in vitro* that PRV-infected monocytes with internalized viral cell surface proteins are indeed significantly less susceptible towards antibody-dependent complement-mediated lysis (ADCML) than infected cells without internalization. *In vivo*, PRV-specific antibodies and complement are simultaneously present in the blood of immune animals whereas in our *in vitro* model, PRV-infected monocytes were first incubated with PRV-specific antibodies for 1h (to allow internalization) and only afterwards incubated with porcine complement. Nevertheless, the process of antibody-induced internalization of viral cell surface proteins in PRV-infected monocytes occurs very rapidly, starting within 5 min post antibody addition (Favoreel *et al.*, 1999), whereas complement needs approximately 10 min to become activated by antibodies (Sissons *et al.*, 1979). Thus, based on our *in vitro* findings, it is to be expected that the process of antibody-induced internalization may be fast enough to

allow PRV-infected monocytes to escape efficient elimination by complement-mediated cell lysis.

Besides ADCML, antibody-dependent cell-mediated cytotoxicity (ADCC) is also a major component of the antibody-dependent immune system. The interaction between antibodies and Fc receptors on cytotoxic effector cells, such as monocytes/macrophages, polymorphonuclear leukocytes, T-cells and natural killer (NK) cells, induces a signal transduction cascade in the effector cells which ultimately leads to destruction of the infected cell (Sissons & Oldstone, 1980). The kinetics of ADCC activation are similar to those of ADCML, but efficient ADCC can already be achieved by a 10-fold lower concentration of antibodies bound to the infected cell (Perrin *et al.*, 1977). Hence, ADCML evasion of PRV-infected monocytes by internalization of the majority of antigen-antibody complexes may not automatically imply efficient ADCC evasion.

Another potential mechanism to escape antibody-dependent components of the immune system has also been described for PRV and for the other alphaherpesviruses herpes simplex virus (HSV) and varicella zoster virus (VZV). This mechanism implies Fc receptor activity of the viral glycoprotein complex gE-gI (Johnson *et al.*, 1988; Litwin *et al.*, 1992; Favoreel *et al.*, 1997). In the current thesis, we showed that PRV gE-gI indeed renders PRV-infected monocytes less susceptible towards ADCML, although with surprisingly low efficiency. A possible explanation for this low efficiency could be that gE-gI specific antibodies interfere with efficient gE-gI Fc receptor activity by binding to the Fc binding domain in the gE-gI complex. In support of this hypothesis, we found that (i) performing the ADCML assay using a PRV-positive pig serum without gE-gI specific antibodies resulted in a much more pronounced protective effect of gE-gI towards ADCML and (ii) based on flow cytometric experiments we showed that gE-gI positive, but not gE-gI negative pig serum, can block gE-gI Fc receptor activity. It would be interesting to further investigate whether, similarly, gE-gI specific antibodies may be able to inhibit Fc receptor activity of HSV and VZV. Also, the possibility that the gE-gI Fc receptor activity may be inhibited through the presence of gE-gI specific antibodies may have consequences regarding vaccination. For PRV, although the currently applied PRV vaccines are certainly efficient and helped in eradicating PRV in several countries, most of the PRV vaccines are based on a gE-deleted mutant. Such a “marker” vaccine

makes it easy to distinguish infected animals (presence of gE-specific antibodies) from vaccinated animals (absence of gE-specific antibodies) (Van Oirschot *et al.*, 1986). Since a gE-negative vaccine fails to elicit a gE-specific antibody response, it can be hypothesized that the PRV gE-gI Fc receptor will not be blocked, which may allow gE-gI-mediated immune-evasion during a subsequent PRV wild type infection. Still, the currently used gE-marker vaccines are successful in eradicating PRV, which may indicate that gE-gI-mediated immune evasion *in vivo* will most likely be of minor importance for PRV.

Since antibody-induced internalization may aid PRV-infected monocytes in masking the cells from efficient immune-mediated destruction (immune-masked monocytes), it is crucial to fully understand the mechanism of this process in order to possibly find methods to interfere with the process. From earlier studies, it is known that the viral proteins gB and gD, present on the cell surface of infected cells, are indispensable for the initiation of the internalization process and only one single tyrosine residue in the cytoplasmic tail of gB is crucial for correct functioning of gB during the internalization process (Favoreel *et al.*, 1999; 2002). To date, it remained unclear if the subsequent steps of internalization are mediated by viral components or rather are directed by the cellular endocytosis machinery. Using different chemical inhibitors and confocal studies, we were able to demonstrate that, after virus-mediated initiation of the process, the subsequent steps of internalization most likely are all physiological and occur via the same route as the well-studied bivalent-ligand induced endocytosis of cellular receptors. Like for the cellular endocytosis process, we showed that during internalization of the antigen-antibody complexes, (i) the cytoskeleton component actin provides the integrity of the cell, necessary for internalization to occur, and clathrin molecules are involved most likely by formation of clathrin-coated pits/vesicles and (ii) microtubules and dynein are necessary for transport of the vesicles towards the perinuclear area of the cell. It is our current thinking that, upon crosslinking induced by the addition of virus-specific antibodies, specific tyrosine-based amino acid motifs in the cytoplasmic tails of viral proteins gB & gD interact with adaptor protein 2 which then link the viral proteins to clathrin-molecules as a first step in the formation of clathrin-coated vesicles. One of the most intriguing questions regarding the mechanism of the internalization process is why the majority of viral cell surface proteins are internalized and not solely the two viral proteins that initiate the process (gB and gD). An attractive hypothesis may be that

viral cell surface proteins are not randomly located in the plasma membrane, but grouped in specialized microdomains in the plasma membrane such as the cholesterol- and sphingolipid-rich lipid rafts (Brown and London, 2000). For several viruses such as human immunodeficiency virus (HIV) and influenza virus, association of viral cell surface proteins with such rafts has already been demonstrated (Scheiffele *et al.*, 1999; Ono & Freed, 2001). Preliminary data suggest that for PRV, at least some plasma membrane-anchored glycoproteins may be associated with or become associated with these microdomains upon antibody crosslinking (H. Favoreel, personal remark). If this would be the case, internalization initiated by gB and gD may result in internalization of the entire microdomain, containing the majority of viral cell surface proteins. Another interesting question is why internalization of viral cell surface proteins does not occur in all PRV-infected monocytes. This observation may perhaps be explained by individually differences in the inherent endocytosis capacity among the heterogeneous population of monocytes. Another explanation may be that different PRV strains have different capacities to induce internalization. Indeed, performing the internalization experiments with different laboratory strains and field strains of PRV resulted in significant differences in the internalization efficiency (ranging from 30% up to 80% of internalization), indicating that the efficiency of internalization is most likely virus-dependent. Since viral proteins gB and/or gD are indispensable for the efficient initiation of internalization, it may be interesting to examine whether the internalization capacity of certain PRV strains may be explained by differences in the gB & gD DNA and amino acid sequences.

In order to be able to transmit virus to internal organs, the immune-masked PRV-infected monocytes do not only have to evade recognition by the immune system, they also have to survive a productive PRV infection long enough to reach these organs. In the current thesis, we observed that a subpopulation of the immune-masked PRV-infected monocytes (but not the non-masked monocytes) survived a PRV infection for remarkable long periods, up to 8 days post inoculation (end of the experiment). Moreover, we found that the majority of these surviving PRV-infected cells did no longer show visually detectable expression of viral proteins, although these cells were productively infected at early stages post inoculation. The most obvious explanation for these observations is that the presence of PRV-specific antibodies leads to a quiescent virus infection in a subpopulation of PRV-infected

monocytes. Suppression of intracellular virus replication upon binding of specific antibodies to infected cells has already been reported for other viruses such as HSV and Sindbis virus (Chanas *et al.*, 1982; Oakes & Lausch, 1984). Binding of antibodies can apparently trigger a signal that leads to a quiescent infection. Interesting in this context is the finding that anti-gB and anti-gE antibodies are most efficient in inducing a quiescent HSV infection (Oakes & Lausch, 1984). Findings at our and other laboratories have indicated that binding of anti-gB and anti-gE antibodies to PRV- and HSV-infected cells can activate cellular tyrosine-phosphorylation dependent signal transduction pathways (Favoreel *et al.*, 1997; 2002; Rizvi & Raghavan, 2003). The combination of these results suggests that antibody-induced activation of intracellular signal transduction pathways may ultimately lead to a quiescent infection. An explanation for the way how activated signal transduction pathways may suppress an ongoing virus infection can perhaps be found when comparing these findings with the knowledge on cytokine-mediated control of virus infections. Binding of several cytokines (e.g. interferons) to infected cells also activates tyrosine-phosphorylation dependent signal transduction pathways which then suppress the infection via multiple ways (Guidotti & Chisari, 2000). Such pathways induce the expression of 2'5'-oligoadenylate synthetases which mediate antiviral activities by induction of a cellular RNAse that degrades viral transcripts, Mx proteins which may bind viral ribonucleoprotein complexes and protein kinase R which may inhibit viral protein synthesis initiation (Guidotti & Chisari, 2000). An attractive hypothesis may therefore be that signal transduction pathways, activated by binding of antibodies to infected cells, may induce similar inhibitory effects on virus replication.

Another interesting *in vitro* finding in our study was that addition of the reactivating agent dexamethasone stimulated a significant fraction of the quiescent PRV-infected cells to resume viral antigen expression. Glucocorticoids, like dexamethasone, have important anti-inflammatory effects but can also reactivate herpesviruses from latency (Schoembaum *et al.*, 1990; Newton, 2000). Whether, based on this observation, it may be hypothesized that this quiescent PRV infection may resemble or lead to a latent PRV infection will depend on further and more extensive experimentations, including *in situ* hybridization to detect latency-associated transcripts. Whereas latency in monocytes/macrophages is a well-known feature of the betaherpesviruses human cytomegalovirus (HCMV) and human

herpesvirus 6 (HHV-6) (Kondo *et al.*, 1991; Sinclair & Sissons, 1996), latency of PRV in blood mononuclear cells is still a matter of debate (Mettenleiter, 1994).

Immune-masked PRV-infected monocytes have to cross the barrier of endothelial cells of maternal blood vessels in order to reach fetal tissues. Adhesion of the immune-masked monocytes to endothelial cells, in the presence of virus-neutralizing antibodies, is a first necessity. We demonstrated that efficient adhesion of immune-masked PRV-infected monocytes to endothelial cells occurs *in vitro* and is mediated by the cellular adhesion molecules wCD11R3 and CD18, and possibly other adhesion molecules such as wCD11R1, wCD11R2 and VLA-4. Following adhesion, we observed a rapid fusion of the adhering infected cells with the underlying endothelial cells, resulting in virus transmission to endothelial cells. The fusion process we observed between immune-masked monocytes and endothelial cells was virus-mediated since the presence of viral glycoprotein gH was indispensable for fusion to occur. Since PRV gH is known to be essential for the well-studied virus-cell fusion process (Peeters *et al.*, 1992b), we suggest that the fusion of immune-masked monocytes with endothelial cells occurs in a similar way. Transmission of virus to endothelial cells via similar (micro)fusion events has already been described for other viruses like HCMV and HIV (Dianzani *et al.*, 1996; Gerna *et al.*, 2000). For the betaherpesvirus HCMV, virus is transferred from polymorphonuclear leukocytes to endothelial cells, and vice versa, via microfusion events which are documented by discontinuation of some areas of the membranes of the adhering cells (Gerna *et al.*, 2000). Thus, based on the results of our *in vitro* study, we demonstrated that adhesion and subsequent fusion of immune-masked monocytes with endothelial cells may provide a means of PRV transmission to endothelial cells. However, other possible routes for PRV to cross the endothelial barrier may not be ruled out at present, with diapedesis of immune-masked PRV-infected monocytes as the most important possible alternative explanation.

Although cell fusion events may explain transfer of PRV from immune-masked monocytes to endothelial cells, it does not explain how PRV subsequently spreads through the different layers of the maternal (and fetal) placenta in order to reach fetal tissues. Further spread of PRV will most likely involve direct cell-to-cell spread, a well-known general feature of herpesviruses. Direct cell-to-cell spread allows the virus to spread from an infected cell to adjacent cells, without entering the (immune-

surveilled) extracellular milieu. This mechanism of direct cell-to-cell spread involves processes which are closely related to the process of virus entry in the cell. For PRV, the difference between these two processes is that gD is essential for virus entry, while it is redundant for cell-to-cell spread (Peeters *et al.*, 1992a). Since abortion in vaccinated sows can still occur upon inoculation of the *arteria uterina* with monocytes infected with a $PRV_{gD\ null}$ mutant (Nauwynck, 1997), it is safe to assume that PRV uses direct cell-to-cell spread to reach the fetal tissues.

In conclusion, the *in vitro* work of this thesis provides strong indications that the process of antibody-induced internalization of viral cell surface proteins on PRV-infected monocytes may be important for PRV to reach internal organs in the presence of an immunity, induced by inactivated vaccines. Moreover, this knowledge may be of importance for further research on the intriguing interactions between blood leukocytes and other herpesviruses like HCMV, EHV-1 and VZV, which are also known to circulate and cause disease in immune hosts (Galil *et al.*, 2002; Revello & Gerna, 2002; van der Meulen *et al.*, 2003).

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7.

SUMMARY/SAMENVATTING

Summary

Pseudorabies virus (PRV) can still replicate in the respiratory tract of the pig in the presence of a vaccination-induced immunity resulting in a restricted viraemia. Moreover, although rare, abortion may be an important consequence of this limited replication in immune animals. Porcine blood monocytes have been shown to be essential to transport the virus throughout the body, including the pregnant uterus, of vaccination-immune pigs. Recently, a potential immune evasion strategy has been proposed how PRV-infected porcine blood monocytes may be able to avoid recognition by the immune system. This process consists of aggregation and subsequent virus-mediated internalization of the majority of antigen-antibody complexes from the cell surface of PRV-infected monocytes.

The aim of the current thesis was to investigate *in vitro* whether this process may lower the susceptibility of the cells towards antibody-dependent cell lysis, what the underlying mechanism of the process is, and how these PRV-infected monocytes with internalized antigen-antibody complexes may transmit virus to internal organs.

In Chapter 1, a brief introduction is given on PRV, with emphasis on pathogenesis of PRV (both in naive and immune pigs) on the one hand and the known PRV immune evasion strategies on the other hand.

In Chapter 2, it was first evaluated whether the process of virus-mediated internalization of antigen-antibody complexes from the cell surface of PRV-infected monocytes indeed has an effect on the efficiency of antibody-dependent cell lysis. Therefore, an *in vitro* model was set up to examine the susceptibility of PRV-infected monocytes towards antibody-dependent complement-mediated lysis (ADCML). PRV-infected monocytes, at 13 h p.i., were incubated for 1 h at 37°C with 0.9 mg/ml FITC-labeled porcine polyclonal PRV-specific antibodies in the presence or absence of 50µg/ml genistein, a tyrosine kinase inhibitor which blocks internalization. In some experiments, the population of infected monocytes with complete internalization were enriched by magnetic cell sorting. Afterwards, cells were incubated with a 5% dilution of porcine complement and finally incubated for 5 min with propidium iodide (PI), which specifically stains dead cells. Fluorescence intensity of the cells was

analyzed by flow cytometry. Approximately 47% of the cells without internalized antigen-antibody complexes was lysed by complement whereas this was only 12% for the cells with internalized complexes, indicating that the internalization process aids in protecting PRV-infected monocytes from antibody-dependent cell lysis. Further, it was studied whether another potential immune evasion mechanism described for several alphaherpesviruses, including PRV, also reduces the susceptibility of PRV-infected monocytes towards antibody-dependent complement-mediated lysis *in vitro*. This immune evasion mechanism consists of the Fc receptor activity of the viral gE-gI complex. This Fc receptor activity may possibly interfere with efficient antibody-dependent cell lysis by 'disarming' the antibodies by binding their Fc region, which normally activates the antibody-dependent immune effectors. Using wild type PRV strains and gE-gI null strains in the ADCML assay, we demonstrated that gE-gI Fc-receptor activity interferes, although at very low efficiency, with efficient ADCML of PRV-infected monocytes. We hypothesized that this low efficiency was due to the fact that some of the polyclonal antibodies in our ADCML assay may have been directed against epitopes on gE and/or gI, important for its Fc receptor function. Such binding can thereby interfere with efficient Fc binding of gE-gI. To support this hypothesis, we repeated the same experiments using porcine PRV-specific antibodies derived from a pig infected with a gE-gI null mutant (and therefore contains no gE-gI-specific antibodies). This resulted in a much more pronounced protective effect of the gE-gI Fc receptor towards ADCML-mediated lysis of PRV-infected monocytes. Further, using flow cytometry, we showed that PRV-specific polyclonal antibodies derived from hyperimmune serum from a wild type virus-inoculated pig (but not from a gE-gI null virus-inoculated pig) were able to block the gE-gI Fc receptor activity. Hence, gE-gI specific-antibodies present in PRV-positive pig serum may be able to inhibit gE-gI Fc receptor functioning. Another interesting observation was that monocytes inoculated with the PRV strain Becker were always 1.5-fold less susceptible towards ADCML than cells inoculated with the PRV strain Kaplan. This was not due to differences in efficiency of internalization of antigen-antibody complexes or gE-gI Fc receptor activity between both strains. Combined with the fact that, even when blocking both antigen-antibody internalization and gE-gI Fc receptor activity, lysis of 100% of PRV-infected monocytes was never achieved, these data

suggest that PRV may have developed additional ADCML evasion mechanisms which could be more effective for certain PRV strains than for others.

In Chapter 3, the underlying mechanism of the antibody-induced internalization of viral cell surface proteins in PRV-infected monocytes was studied in more detail. Up till now, only the initiation of this process had been unraveled and was shown to be mediated by the viral proteins gB and gD, expressed on the cell surface of PRV-infected monocytes. Further research was necessary to clarify whether the subsequent steps of the internalization process were also mediated by the virus or merely involved the cellular endocytosis machinery. The cellular components, known to play a role during physiological endocytosis processes, have already been extensively studied. The cellular cytoskeleton component actin is important during the first stages of endocytosis, together with clathrin which mediates membrane invagination and vesicle formation. For further transport of vesicles towards the center of the cell, microtubules and the motor protein dynein are indispensable. In this chapter, the involvement of cellular cytoskeleton components during different steps of the internalization of PRV cell surface proteins was investigated via two ways. On the one hand, PRV-infected monocytes were treated with different concentrations of chemical inhibitors for 45 min before and also during the addition of PRV-specific antibodies. The chemical inhibitors used were colchicine, cytochalasin D, latrunculin B and amantadine-HCl, known to inhibit polymerization of microtubules, actin/clathrin, actin and clathrin respectively. Addition of each chemical inhibitor resulted in a significant reduction of cells with internalized viral proteins compared to non-treated controls. On the other hand, to further elucidate the role of the different cellular components during internalization, double labeling experiments of viral proteins and cellular components were performed during the different redistribution stages of the internalization process. A clear co-localization was observed between the viral glycoproteins and clathrin during the early stages of the internalization process. No change in the distribution of actin could be observed, implying a more passive role for actin. A clear co-localization between the viral glycoproteins and dynein was observed during the later stages internalization and the internalized vesicles were in close contact with microtubules. Hence, these data demonstrate that upon the viral-induced initiation of the internalization process, all further steps involve cellular

components, in a similar way as observed during physiological receptor-mediated endocytosis processes.

Besides the sensitivity towards recognition by the immune system another factor determining the chance of an PRV-infected monocyte to reach internal organs, is the time the monocyte may survive the infection. Indeed, a productive PRV infection results rapidly in cell death, which would obscure the chances of the virus to reach internal organs. Therefore, in Chapter 4, *in vitro* studies were performed to investigate the long-term fate of the PRV-infected monocytes with internalized viral proteins (immune-masked monocytes) with regard to viability and expression of viral antigens. In a first assay, the viability of PRV-infected monocytes in the presence or absence of PRV-specific antibodies was evaluated until 194 h p.i. In the continuous presence of PRV-specific antibodies, approximately 30% of the PRV-infected monocytes were still viable at 194 h p.i., compared to 0% in the absence of PRV-specific antibodies. These results demonstrate that in the presence of PRV-specific antibodies, some PRV-infected monocytes survive for unusual long periods of time. In a second assay, the expression of viral glycoproteins was investigated in the population of immune-masked monocytes which survived a PRV infection for unusual periods of time (in the continuous presence of PRV-specific antibodies). Although all PRV-infected monocytes showed viral protein expression at 96 h p.i., approximately 75% of the surviving immune-masked PRV-infected monocytes did no longer show visually detectable expression of viral proteins from 120 h p.i. onwards, in the continuous presence of PRV-specific antibodies. This implies that a subpopulation of PRV-infected cells developed a quiescent infection status. Addition of dexamethasone (an agent known to reactivate herpesviruses from latency) was able to induce a re-expression of viral proteins. The results of this study demonstrated that antibody-induced internalization of viral proteins leads to an expanded life span of the cells and suppression of viral protein expression in a subpopulation of the PRV-infected monocytes, which may help explaining how PRV-infected monocytes can act as carriers of the virus to internal organs in the blood of vaccinated animals.

In Chapter 5, it was shown *in vitro* that the immune-masked PRV-infected monocytes are able to transmit PRV to endothelial cells of blood vessels in the

presence of virus-neutralizing antibodies by means of adhesion and fusion processes. To this end, porcine blood monocytes were inoculated with a LacZ-carrying PRV strain and at 13 h p.i. incubated with FITC-labeled PRV-specific antibodies to induce internalization of the viral cell surface proteins. Afterwards, these cells were cocultivated with monolayers of cultured porcine endothelial cells in the presence of virus-neutralizing antibodies. At different time points, monolayers of endothelial cells were fixed and stained with X-gal to visualize LacZ expression. It was demonstrated that the adhesion of monocytes to endothelial cells reached its plateau at 60 min of cocultivation and that PRV-infected monocytes with internalized viral cell surface proteins (immune-masked) adhere much more efficiently to endothelial cells than PRV-infected monocytes without internalization (50% adhesion versus 4% adhesion respectively). When evaluating the LacZ transmission from the cytoplasm of the infected monocyte to the underlying endothelial cells, as a measure for monocyte/endothelial cell fusion, it was found that fusion started from 30 min of cocultivation and reached a level of 38.0% ± 2.5 of fused immune-masked monocytes at 120 min of cocultivation. Virus transmission during the fusion process was confirmed by the appearance of plaques. Performing double immunofluorescence stainings for some of the most important adhesion molecules expressed on monocytes on the one hand and viral cell surface proteins on the other hand, in combination with an adhesion blocking assay, demonstrated that the adhesion of immune-masked monocytes to endothelial cells was mediated by the cellular adhesion molecules wCD11R3 and CD18. Using a LacZ-carrying PRV strain deleted in the gene encoding the viral protein gH, essential for virus-cell fusion and cell-to-cell spread, resulted in immune-masked PRV-infected monocytes that were unable to fuse with endothelial cells. This indicates that fusion is virus-mediated, probably by a mechanism similar to virus-cell fusion. The adhesion and fusion processes described here may possibly explain how PRV crosses the endothelial cell barrier of placental blood vessels in vaccinated animals, as a first step to reach the pregnant uterus.

As a general conclusion, based on the current *in vitro* findings, it can be stated that the process of antibody-induced internalization of viral cell surface proteins in PRV-infected monocytes is likely to be an important mechanism used by PRV to circulate in the blood of vaccinated animals. The process, initiated by the virus and completed by the cellular endocytosis machinery, may enable PRV to evade the antibody-

dependent components of the immune system. Further, it allows PRV-infected monocytes to survive the infection for unusual long periods of time in the presence of PRV-specific antibodies, most likely by inducing an abortive infection. Finally, the internalization process allows PRV-infected monocytes to efficiently adhere and transmit virus to endothelial cells in the presence of neutralizing antibodies and this may be an important first step for the virus to reach internal organs in vaccinated animals.

Samenvatting

Het Aujeszky virus (AV), ook wel pseudorabies virus genoemd, kan vermeerderen in het ademhalingsstelsel van varkens, geïmmuniseerd met geïnactiveerde vaccins, wat kan resulteren in een beperkte viremie. Als gevolg van deze beperkte viremie kan AV-geïnduceerde abortus, weliswaar zeldzaam, nog voorkomen bij immune zeugen (gevaccineerd met een geïnactiveerd vaccin). Geïnfecteerde bloed monocyten zijn essentieel voor het transport van het virus naar de drachtige baarmoeder in deze vaccinatie-immune dieren. Een mogelijk mechanisme dat zou kunnen verklaren hoe AV-geïnfecteerde monocyten ontsnappen aan efficiënte herkenning en eliminatie door het immuunsysteem werd recentelijk naar voren gebracht. Dit proces bestaat erin dat na binding van antistoffen op de virale antigenen die aanwezig zijn op de plasmamembraan van de geïnfecteerde monocyt, deze antigeen-antistofcomplexen aggregeren en snel door de cel opgenomen worden (internalisatie). De internalisatie van de antigeen-antistofcomplexen wordt geïnduceerd door twee virale eiwitten op de plasmamembraan, namelijk gB en gD.

Het doel van deze thesis was om *in vitro* na te gaan of (i) dit internalisatieproces de gevoeligheid van AV-geïnfecteerde monocyten tegen antistof-afhankelijke cellyse inderdaad kan verminderen, (ii) wat het onderliggend mechanisme is van het internalisatieproces en (iii) op welke manier AV-geïnfecteerde monocyten met geïnternaliseerde antigeen-antistofcomplexen virus kunnen overdragen naar endotheelcellen van de bloedvatwand.

In Hoofdstuk 1 werd een korte inleiding gegeven over AV, met de nadruk op de pathogenese in zowel naïeve als immune varkens. Daarnaast werden de immuno-evasie mechanismen die reeds bekend zijn voor AV uitgebreid besproken.

In Hoofdstuk 2 werd eerst en vooral onderzocht of het proces van virus-gemedieerde internalisatie van antigeen-antistofcomplexen op de celmembraan van AV-geïnfecteerde monocyten inderdaad een effect heeft op de doeltreffendheid van antistof-afhankelijke cellyse. Een *in vitro* model werd geconstrueerd om de gevoeligheid van AV-geïnfecteerde monocyten tegen antistof-afhankelijke complement-gemedieerde cellyse (AACGL) na te gaan. AV-geïnfecteerde

monocyten, op 13 uren na inoculatie, werden geïncubeerd met 0,9 mg/ml FITC-gemerkte polyklonale AV-specifieke varkensantistoffen gedurende 1 uur bij 37°C in de aan- of afwezigheid van 50µg/ml genisteïne, een specifieke inhibitor van het internalisatieproces. In bepaalde experimenten werd de populatie van geïnfecteerde monocyten met geïnternaliseerde antigeen-antistofcomplexen aangerijkt door middel van magnetische celsortering. Nadien werden de cellen geïncubeerd gedurende 1 uur met een 5% verdunning van varkenscomplement en tenslotte geïncubeerd gedurende 5 minuten met het fluorescerende propidiumjodide dat specifiek DNA van dode cellen merkt. De fluorescentie-intensiteit van de cellen werd nagegaan aan de hand van flowcytometrie. Ongeveer 47% van de cellen zonder internalisatie werden gelyseerd door complement in tegenstelling tot slechts 12% van de cellen met geïnternaliseerde antigeen-antistofcomplexen. Dit resultaat toont aan dat het internalisatieproces van antigeen-antistofcomplexen kan helpen bij de bescherming van AV-geïnfecteerde monocyten tegen antistof-afhankelijke cellyse *in vivo*. Daarnaast werd onderzocht of een ander immuno-evasie mechanisme, reeds beschreven voor verschillende alfaherpesvirussen waaronder AV, ook in staat is de gevoeligheid van AV-geïnfecteerde monocyten te verminderen tegen AACGL. Dit immuno-evasie mechanisme bestaat uit de Fc-receptoractiviteit van het virale eiwitcomplex gE-gI dat aanwezig is op de plasmamembraan van geïnfecteerde cellen. Deze Fc-receptoractiviteit kan interfereren met doeltreffende antistof-afhankelijke cellyse. Door binding van gE-gI met de Fc-regio van antistoffen, kan deze Fc-regio de antistof-afhankelijke immuuncomponenten niet langer activeren. AACGL experimenten met AV wild type stammen en AV mutanten die de genen missen die coderen voor het gE-gI eiwitcomplex toonden aan dat monocyten geïnfecteerd met wild type AV iets minder gevoelig waren voor AACGL dan monocyten geïnfecteerd met de gE-gI-negatieve AV stam. Met andere woorden, de gE-gI Fc-receptoractiviteit beschermt de AV-geïnfecteerde monocyten enigszins tegen AACGL, maar op een weinig efficiënte manier. Een mogelijke hypothese voor dit weinig efficiënt effect van de gE-gI Fc-receptor is dat sommige antistoffen in het AV-specifiek polyklonaal serum dat gebruikt wordt tijdens de AACGL experimenten, mogelijk gericht zijn tegen de epitopen op gE-gI die belangrijk zijn voor de Fc-receptoractiviteit. Daardoor kunnen deze IgG-bindende epitopen op gE-gI bedekt zijn met antistoffen waardoor ze hun Fc-receptoractiviteit niet kunnen uitvoeren. Om deze hypothese te ondersteunen

werden dezelfde AACGL experimenten herhaald, maar nu met AV-specifieke varkensantistoffen die afkomstig waren van een varken dat geïnfecteerd was met een AV gE-gI-negatieve mutant (en dat bijgevolg geen gE-gI-specifieke antistoffen heeft). Dit resulteerde in een duidelijk meer uitgesproken beschermend effect van de gE-gI Fc-receptor tegen AACGL van AV-geïnfecteerde monocyten. Bovendien konden we aantonen, aan de hand van flowcytometrie, dat AV-specifieke polyklonale antistoffen afkomstig van een varken geïnoculeerd met wild type AV virus (maar niet van een varken geïnoculeerd met een AV gE-gI-negatieve mutant) in staat waren de gE-gI Fc-receptoractiviteit te blokkeren. Samengevat kunnen we stellen dat gE-gI-specifieke antistoffen aanwezig in AV-positief varkensserum waarschijnlijk in staat zijn de gE-gI Fc-receptorfunctie te inhiberen. Een andere interessante observatie was dat monocyten geïnoculeerd met de AV stam Becker altijd 1,5 maal minder gevoelig waren tegen AAGCL dan wanneer cellen geïnoculeerd waren met de AV stam Kaplan. Dit was niet te wijten aan verschillen in de onderzochte immuno-evasie mechanismen (de internalisatie van antigeen-antistofcomplexen en de gE-gI Fc-receptoractiviteit) tussen beide stammen. Gecombineerd met het feit dat in onze experimenten AACGL nooit in staat was alle AV-geïnfecteerde monocyten te lyseren, zelfs indien zowel antigeen-antistof internalisatie als gE-gI Fc-receptoractiviteit geïnhibeerd werden, suggereren deze gegevens dat AV mogelijk andere AACGL-evasie mechanismen ontwikkeld heeft die waarschijnlijk efficiënter zijn voor sommige AV stammen dan voor andere.

In Hoofdstuk 3 werd het onderliggend mechanisme van het internalisatieproces van antigeen-antistofcomplexen in AV-geïnfecteerde monocyten bestudeerd. Tot op heden was enkel opgehelderd dat de virale eiwitten gB en gD, die aanwezig zijn op de plasmamembraan van AV-geïnfecteerde monocyten, noodzakelijk zijn om het internalisatieproces te initiëren. Bijkomend onderzoek was nodig om te achterhalen of ook de volgende stappen van het internalisatieproces gemedieerd zijn door het virus of dat hiervoor eerder de cellulaire endocytose machinerie verantwoordelijk is. De cellulaire componenten die een belangrijke rol spelen tijdens fysiologische endocytose processen zijn reeds uitgebreid bestudeerd. De cellulaire component actine is vooral belangrijk tijdens de eerste stappen van de endocytose, samen met clathrine die de membraaninvaginatie en de vorming van vesikels medieert. Voor verder transport van deze vesikels in de richting van de celkern zijn microtubuli en de

motorproteïne dyneine onontbeerlijk. In dit hoofdstuk werd op twee manieren onderzocht of de cellulaire cytoskelet componenten een rol spelen tijdens de verschillende stappen van internalisatie. Hiervoor werden in eerste instantie monocyten, op 13 uren na inoculatie met AV, behandeld met verschillende inhibitoren gedurende 45 minuten voor en ook tijdens de toediening van AV-specifieke antistoffen. Deze inhibitoren waren colchicine, cytochalasine D, latrunculine B en amantadine-HCl, bekend voor hun inhiberende werking op de polymerisatie van microtubuli, actine/clathrine, actine en clathrine respectievelijk. Toediening van elk van deze inhibitoren resulteerde in een significante daling van het aantal AV-geïnfecteerde cellen met geïnternaliseerde antigeen-antistofcomplexen in vergelijking met de niet-behandelde controlecellen. Om de rol van de verschillende cellulaire componenten tijdens internalisatie verder uit te diepen, werden daarnaast ook immunofluorescentie-dubbelkleuringen uitgevoerd voor virale eiwitten enerzijds en cellulaire componenten anderzijds gedurende de verschillende stadia van het internalisatieproces. Een duidelijke co-localisatie van virale eiwitten met clathrine werd gezien tijdens de eerste fasen van het internalisatieproces. De distributie van actine filamenten bleef gedurende het volledige internalisatieproces onveranderd, waardoor actine waarschijnlijk een meer passieve rol heeft tijdens het proces. Een duidelijke co-localisatie van virale eiwitten met dyneine werd gezien tijdens de latere fases van het internalisatieproces en de geïnternaliseerde vesikels hadden nauw contact met de microtubuli. Samengevat tonen deze resultaten aan dat na de virus-gemedieerde initiatie van het internalisatieproces (via gB en gD), de verdere stappen gebeuren met behulp van cellulaire componenten en dit op een manier gelijkaardig aan de fysiologische receptor-gemedieerde endocytose processen.

Naast de gevoeligheid voor herkenning door het immuunsysteem is er nog een factor belangrijk in de bepaling of een AV-geïnfecteerde monocyt kans maakt om interne organen te bereiken, namelijk de tijd dat een monocyt de infectie kan overleven. Normaal gezien leidt een AV infectie snel tot celdood waardoor de kans vermindert dat het virus interne organen kan bereiken. Daarom werden er in Hoofdstuk 4 *in vitro* experimenten uitgevoerd om het lot van AV-geïnfecteerde monocyten met geïnternaliseerde antigeen-antistofcomplexen (immuun-gemaskeerde monocyten) na te gaan op langere termijn, meer bepaald door de vitaliteit van de

cellen en de expressie van virale eiwitten in deze cellen nader te bekijken. In een eerste proefopzet werd de vitaliteit van AV-geïnfecteerde monocyten nagegaan, zowel in de aan- als afwezigheid van AV-specifieke antistoffen, tot 194 uren na inoculatie. In de aanwezigheid van AV-specifieke antistoffen was 194 uren na inoculatie nog ongeveer 30% van de geïnfecteerde monocyten levend in tegenstelling tot 0% levende AV-geïnfecteerde cellen in de afwezigheid van AV-specifieke antistoffen. Deze resultaten tonen aan dat bepaalde AV-geïnfecteerde monocyten voor ongewoon lange tijd overleven in de aanwezigheid van AV-specifieke antistoffen. In een tweede proefopzet werd de expressie van virale eiwitten nagegaan in de populatie van AV-geïnfecteerde monocyten die een AV infectie ongewoon lang overleefden (in de aanwezigheid van AV-specifieke antistoffen). Alhoewel alle AV-geïnfecteerde monocyten virale eiwitten tot expressie brachten, 96 uren na inoculatie, vertoonde ongeveer 75% van de levende immuun-gemaskeerde AV-geïnfecteerde cellen geen visueel detecteerbare expressie meer van virale eiwitten vanaf 120 uren na inoculatie. Dit toont aan dat onder invloed van de AV-specifieke antistoffen, een subpopulatie van AV-geïnfecteerde monocyten een abortieve infectie ontwikkelde. Toediening van dexamethasone (een corticosteroïd dat bekend is om herpesvirussen uit latentie te reactiveren) was in staat een re-expressie van virale eiwitten te induceren. Deze studie toont aan dat internalisatie van antigeen-antistofcomplexen resulteert in een verlengde overlevingstijd van de cellen en een onderdrukken van de expressie van virale eiwitten in een subpopulatie van de AV-geïnfecteerde monocyten, wat mogelijk helpt verklaren hoe AV-geïnfecteerde monocyten dragers kunnen zijn van het virus in het bloed van gevaccineerde dieren.

In Hoofdstuk 5 tenslotte werd aangetoond dat de immuun-gemaskeerde AV-geïnfecteerde monocyten in staat zijn om het virus over te dragen naar endotheelcellen van bloedvaten *in vitro* in de aanwezigheid van virus-neutraliserende antistoffen en dit via adhesie- en fusieprocessen. Om dit aan te tonen werden varkensmonocyten geïnoculeerd met een LacZ-dragende AV stam en 13 uren later werden FITC-gemerkte AV-specifieke antistoffen toegediend om internalisatie van de antigeen-antistofcomplexen te verkrijgen (immuun-gemaskeerde monocyten). Nadien werden de monocyten in cultuur gebracht met een monolaag van gecultiveerde endotheelcellen in aanwezigheid van virus-neutraliserende antistoffen. Vervolgens werden de monolagen op verschillende tijdstippen gefixeerd en gekleurd met X-gal

om LacZ expressie te visualiseren. Adhesie van AV-geïnfecteerde monocyten aan endotheelcellen bereikte een plateau op 60 minuten na co-cultivatie en enkel de immuun-gemaskeerde monocyten (dus deze met geïnternaliseerde antigeen-antistofcomplexen) waren in staat efficiënt te adhereren aan endotheelcellen (ongeveer 50% in vergelijking met slechts 4% voor monocyten zonder internalisatie). De LacZ overdracht van het cytoplasma van de geïnfecteerde monocyt naar het cytoplasma van het onderliggend endotheel werd gebruikt als een maat voor monocyt/endotheelcel fusie. Fusie van immuun-gemaskeerde AV-geïnfecteerde monocyten met endotheelcellen werd waargenomen vanaf 30 minuten na co-cultivatie en bereikte een maximum van 38.0% ± 2.5 gefusioneerde monocyten, 120 minuten na co-cultivatie. De transmissie van het virus werd bevestigd door het verschijnen van plaques in de monolagen van endotheelcellen. Immunofluorescentie-dubbelkleuringen van virale eiwitten enerzijds en enkele van de belangrijkste adhesie moleculen die aanwezig zijn op de monocyten anderzijds, aangevuld met een adhesie-inhibitie proefopzet, toonden aan dat adhesie van immuun-gemaskeerde AV-geïnfecteerde monocyten met endotheel gemedieerd werd door de cellulaire adhesiemoleculen wCD11R3 en CD18. Monocyten geïnfecteerd met een LacZ-dragende AV mutant die het gen mist dat codeert voor het virale eiwit gH (dat essentieel is voor virus-cel fusie en cel-tot-cel spreiden van het virus) waren nog in staat te adhereren aan maar niet langer te fusioneren met endotheelcellen. Dit toonde aan dat de fusie virus-gemedieerd is, hoogstwaarschijnlijk via een mechanisme gelijkaardig aan dat van virus-cel fusie. Samengevat, adhesie van immuun-gemaskeerde AV-geïnfecteerde monocyten aan en fusie met endotheelcellen kunnen mogelijk verklaren hoe AV in staat is de endotheelbarrière van placentale bloedvaten bij gevaccineerde dieren te doorkruisen, als een eerste stap om de drachtige baarmoeder te bereiken.

Als algemene conclusie, gebaseerd op de huidige *in vitro* bevindingen, kan gesteld worden dat antistof-geïnduceerde internalisatie van antigeen-antistofcomplexen in AV-geïnfecteerde monocyten hoogstwaarschijnlijk een belangrijk mechanisme is dat AV mede in staat stelt te circuleren in het bloed van immune dieren, gevaccineerd met geïnactiveerde vaccins. Dit proces, geïnitieerd door het virus en vervolledigd door de cellulaire endocytose machinerie, laat AV-geïnfecteerde monocyten toe om te ontsnappen aan antistof-afhankelijke componenten van het immuunsysteem.

Bovendien kunnen deze immuun-gemaskeerde AV-geïnfecteerde monocyten een infectie voor ongewoon lange tijd overleven, waarschijnlijk doordat onder invloed van de AV-specifieke antistoffen de vermeerdering van AV in deze cellen wordt onderdrukt. Tenslotte zorgt het internalisatieproces er ook voor dat AV-geïnfecteerde monocyten efficiënt kunnen adhereren aan en virus overbrengen naar endotheelcellen in de aanwezigheid van neutraliserende antistoffen, wat een belangrijke eerste stap kan zijn voor AV om interne organen van gevaccineerde dieren te bereiken.

CURRICULUM VITAE

PERSONALIA

Gerlinde Rembrand André Jeanette Van de Walle werd op 16 augustus 1975 geboren te Gent. In 1993 beëindigde zij haar humaniora-opleiding aan het Sint-Pietersinstituut te Gent, richting Latijn-Wetenschappen (A.S.O.). In 1999 werd het diploma dierenarts behaald aan de Faculteit Diergeneeskunde van de universiteit Gent met onderscheiding. Vanaf januari 2000 tot op heden beschikte zij over een doctoraatsbeurs van het Bijzonder Onderzoeksfonds van de Universiteit Gent die kaderde in een Geconcerteerde Onderzoeks Activiteit (GOA) aan het Laboratorium voor Virologie, Vakgroep Virologie, Parasitologie en Immunologie aan de Faculteit Diergeneeskunde, Universiteit Gent. Dit onderzoek handelde over de mogelijke rol van immuno-gemaskeerde Aujeszky virus-geïnfecteerde monocyten tijdens het circuleren van het Aujeszky virus in gevaccineerde varkens en gaf aanleiding tot een 5-tal publicaties, waarvan er reeds 4 gepubliceerd zijn in internationale tijdschriften.

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